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Extracellular Enzyme Synthesis in the Genus *Bacillus*

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Enzymes almost exclusively derived from microorganisms will surely become a multibillion dollar industry in the 70's. They will be the major growth products of the fermentation industry. – A. E. Humphrey, 1972.

INTRODUCTION

As the decade progresses this prediction is rapidly being realized, and microbial enzymes are becoming increasingly important in such diverse fields as medicine, brewing, and timber preservation. The genus *Bacillus* has played a major role in this development as evidenced by the distribution of the papers read at the Fifth International Fermentation Symposium, 1976 (64). Of 23 papers in the session devoted to "Microbial Enzymes of Industrial Interest" no less than ten were concerned with enzymes from bacilli. Reasons for the predominance of these bacteria in this area of study are several. First, they comprise a group of chemoorganotrophs that can be easily maintained and cultivated and yet are markedly heterogeneous in character. Psychrophiles, mesophiles, and thermophiles, in addition to alkalophilic, neutrophilic, and acidophilic species are well represented. Furthermore, virtually all 48 species of the genus listed in *Bergey's Manual of Determinative Bacteriology* (92) secrete a variety of soluble extracellular enzymes, which reflects the diversity of the parental habitats. Amylases that can liquefy starch under pressure at 110°C (194) and proteases that are stable and active at pH 12.0 (6) are extreme examples of enzyme adaption.

This article will attempt to review the recent literature concerned with the characterization and properties of the exoenzymes synthesized by the bacilli and the control and mechanisms of their synthesis. It is restricted to this genus because the commercial importance of extracellular enzymes and academic interest in the process of sporulation have prompted a considerable amount of research into this general area. Nevertheless, in the final section I have attempted to equate our present knowledge of exoenzyme synthesis in procaryotes other than

bacilli with the process as we understand it in *Bacillus* species to outline any unifying concepts.

It has been 14 years since the last article of this kind (247), but relevant reviews concerned with the commercial applications of exoenzymes (36, 86, 335), the significance of these enzymes in the process of sporulation (71, 196, 280), the transport of proteins across membranes (176), and the environmental control of exocellular macromolecule synthesis (35) have been published in the interim.

CELLULAR LOCATION

Extracellular or exoenzymes are those enzymes that are completely dissociated from the cell and found free in the surrounding medium. However, the division between these and cell wall or membrane-bound enzymes is often narrow. Enzymes may be membrane bound in young cells and released as exoenzymes as the culture enters stationary phase (39) or solubilized by relatively mild procedures including washing the cells with water (39) or concentrated salt solution (339).

The periplasm of gram-negative bacteria is recognized as an enzyme-containing compartment bounded on the inside by the cytoplasmic membrane and on the outside by the outer membrane of the cell wall (101). The absence of an outer membrane in the gram-positive cell wall precludes the possibility of a comparable location. Nevertheless, enzymes from bacilli are often described as "periplasmic" in that the procedures adopted in the laboratory to release them are similar to those used for the preparation of periplasmic enzymes from gram-negative sources. Thus a deoxyribonuclease (DNase) (17), 5'-nucleotidase (222), and nucleoside diphosphate-sugar hydrolase (203) may be released from *B. subtilis* strains by protoplasting. The exact location of these enzymes and their relationship to truly extracellular enzymes remain obscure, but it should be noted that at least some exoenzymes do have a periplasmic counterpart which may be released by protoplasting (229, 272, 273).

Exoenzymes are not synthesized simultaneously as cytoplasmic and extracellular molecules. There are three lines of evidence that suggest that at no time do exoenzymes occur within the cytoplasm. First, the intracellular levels of α-amylase and protease in *B. amyloliquefaciens* are virtually negligible and any activity that can be detected probably represents trapped exoenzyme (50, 204, 229). Second, there exists in the cytoplasm of *B. amyloliquefaciens* an inhibitor of extracellular ribonuclease (RNase) (296). Since the formation of the enzyme inhibitor complex is essentially irreversible (114), it seems unlikely that the native enzyme could exist within the cell. Finally, there is a close coupling between the synthesis of exoenzymes and their secretion, suggesting that there is no sizable "pool" of internal enzyme (23).

However at least two exoenzymes, penicillinase and α-amylase, can be detected as both membrane-bound and truly extracellular molecules. At neutral pH approximately half of the penicillinase synthesized by *B. licheniformis* is secreted into the environment in a very stable hydrophilic form (272). Of the remaining cell-bound enzyme, 50% is tightly associated with the cytoplasmic membrane and 50% is associated with periplasmic vesicles (273). The cell-bound penicillinase can be released by limited trypsin digestion (175) or incubation of the cells at pH 9.0 (274). Alternatively, it can be solubilized with a combination of detergent (taurodeoxycholate) and a chelating agent (278). The cell-bound enzyme released by trypsin digestion differs from exopenicillinase only by the absence of the NH_2-terminal lysine residue (2, 210), and, when solubilized by detergent instead of protease, it has been found to be identical to the exoenzyme by amino acid analysis (277). Genetic studies have revealed a single structural gene for penicillinase in *B. licheniformis* (292) from which, presumably, both enzymes are derived. Furthermore, the two enzymes are kinetically very similar (175), have similar substrate specificities, and are identical as determined by radioimmune inhibition assay and immunodiffusion in agar (275). Despite these similarities, the properties of the two forms of the enzyme in solution differ greatly. The exopenicillinase is a highly soluble molecule that does not adsorb to the cell membrane or tend to aggregate (275). The membrane-bound penicillinase, on the other hand, is very hydrophobic in nature. It is found tightly associated with the cytoplasmic membrane and, when solubilized, readily aggregates with itself or other membrane proteins and forms aggregates with ionic and nonionic detergents (175). It has a lower mobility in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (56). Sawai and Lampen (278) purified the membrane penicillinase from *B. licheniformis* grown in media containing $H_3{}^{33}PO_4$ or [^{3}H]glycerol and found it to be correspondingly labeled. Exoenzyme prepared from cells grown in the same medium was devoid of label. Subsequently, the membrane penicillinase has been purified and characterized (344). It differs from the exoenzyme in carrying an addi-

tional phospholipopeptide chain of 25 amino acids. The amino acids are relatively polar, the hydrophobic character of the molecule being derived from an NH_2-terminal phosphatidyl serine residue. The possible role of this membrane-bound penicillinase in the process of enzyme secretion is discussed below.

Evidence is now accumulating to suggest that α-amylase also exists as a membrane-associated and as an extracellular enzyme (229, 260). Nagata et al. (229) have described cell-bound α-amylase in a variety of strains including *B. subtilis* Marburg, *B. natto*, *B. amyloliquefaciens*, and *B. subtilis* var. *amylosaccharitiicus*. Both the extracellular and cell-bound α-amylases of *B. subtilis* are derived from the same structural gene, and point mutations in *amyE* (the structural gene for α-amylase) prevent the synthesis of both forms of the enzyme. The cell-bound α-amylase can be separated into three fractions by chromatography on Sephadex G-75. These correspond to native extracellular enzyme, believed to represent molecules en route to the exterior, and two higher-molecular-weight species which consist of enzyme associated with membrane fragments.

About 50% of the cell-bound penicillinase of *B. licheniformis* is located in periplasmic vesicles (273). These tubular and vesicular membranous structures are induced in *B. licheniformis* by cephalosporin and released into the medium by protoplasting, thus discharging their contents. Such characteristic organelles have not been reported for other exoenzyme-synthesizing systems and their role in penicillinase secretion remains obscure, particularly since they have been shown to be nonessential (176).

SURVEY OF EXOENZYMES

The large variety of exoenzymes synthesized by the bacilli is summarized in Table 1. The amylases and proteases are probably the most ubiquitous, and, of the 48 *Bacillus* species listed in *Bergey's Manual of Determinative Microbiology* (92), 32 degrade starch and a large proportion degrade casein.

α-Amylase hydrolyzes the α-1,4-glucosidic bonds in starch and related polysaccharides. Two principal types of α-amylase are produced by the bacilli, the liquefying and saccharifying enzymes (201). They can be distinguished primarily by their action on starch, the increase in reducing power produced by the saccharifying enzyme being about twice that of the liquefying enzyme. *B. subtilis* var. *amylosaccharitiicus* and *B. natto* are closely related to *B. subtilis* Marburg, and all three produce various amounts of the saccharifying α-amylase (201, 348). In this article they will be referred to as *B. subtilis* SAC, *B. subtilis* NAT, and *B. subtilis*, respectively. *B. amyloliquefaciens* is more distantly related to *B. subtilis* Marburg and secretes large amounts of liquefying α-amylase (286, 332, 348). Traditionally, α-amylase and the cyclodextrin glucosyltransferase of *B. macerans* were thought to be the only starch-degrading enzymes produced by the bacilli, but recently β-amylases (119, 199, 261, 295) and α-1,6-hydrolyzing, starch-debranching enzymes (104, 234, 322, 323) have been detected in various species. Other extracellular carbohydrases from the bacilli include cellulases, hemicellulases, and a variety of β-glucanases (Table 1).

There is a widespread distribution of pectinolytic activity throughout the genus (174, 243). It seems likely that the predominant extracellular pectinolytic enzyme from *Bacillus* species is endopolygalacturonic acid *trans*-eliminase (60, 61, 149, 231), an enzyme that cleaves polygalacturonic acid in a random manner to yield unsaturated digalacturonic or trigalacturonic acid, depending on the species from which the enzyme was obtained.

Microbial proteases have been divided into four groups on the basis of mechanism of action (224). These are (i) serine proteases; (ii) metal (or metal-chelator sensitive) proteases; (iii) thiol proteases; and (iv) acid proteases. With very few exceptions (see Table 1), the extracellular proteases of the genus *Bacillus* are either serine or metal enzymes. A great deal is known of the chemistry and structure of these enzymes, subjects that have been recently reviewed (169, 198, 200) and which lie outside the scope of this article.

The serine proteases have molecular weights in the 25,000 to 30,000 range and are characterized by the presence of a serine residue at their active site. Accordingly, they are inhibited by diisopropyl-fluorophosphate but, possessing no metal ion requirement, they are resistant to ethylenediaminetetraacetic acid (EDTA). (They are, however, stabilized at a high temperature by Ca^{2+}.) They hydrolyze simple terminal esters and, in general, resemble the animal enzymes trypsin and chymotrypsin. The alternative name, alkaline protease, reflects their high pH optimum of 9.0 to 11.0. The serine proteases can be divided into two groups on the basis of their general properties. Group A is typified by the enzymes from *B. licheniformis* (subtilisin Carlsberg [6]) and *B. pumilus*; and group B serine proteases are related to subtilisin Novo, which is identical to subtilisin BPN (297), the enzyme from *B. amyloliquefaciens*.

TABLE 1. *Extracellular enzymes of the genus Bacillus*

Enzyme	Species	Comments	Reference
Carbohydrases			
Agarase	*Bacillus* sp.	Hydrolyzes the β-1,4 linkage of agarose	324
α-Amylase	*B. amyloliquefaciens*	Endohydrolysis of the α-1,4-glucosidic linkages in polysaccharides; the different species produce enzymes with different properties	201, 202, 334
	B. caldolyticus		105, 117
	B. coagulans		18
	B. licheniformis		211, 268
	B. macerans		177, 314
	B. stearothermophilus		237, 245
	B. subtilis		201, 202
	B. subtilis var. *amylosachariticus*		88, 201, 202
	Alkalophilic *Bacillus* spp.		25, 126, 343
β-Amylase	*B. cereus*	Exohydrolysis of the α-1,4-glucosidic linkages in polysaccharides yielding β-maltose	295
	B. megaterium		119
	B. polymyxa		103, 104, 199
	Alkalophilic *Bacillus* spp.		25
Arabinase	*B. subtilis*	Probably an endo α-1,5-arabinase	145
Cellulase	*B. brevis*	Hydrolysis of carboxymethyl cellulose to cellobiose	166
	B. firmus		166
	B. polymyxa		84, 99
	B. pumilus		166
	B. subtilis		166
Chitinase	*B. circulans*	Four enzymes induced by growth on crab-shell chitin	310
Chitosanase	*Bacillus* sp. R-4	Hydrolyzes cell walls of *Rhizopus* spp.	318
Cyclodextrin glucanotransferase	*B. macerans*	Synthesizes cyclo-(Schardinger) dextrins from starch	67, 177
	B. megaterium		162, 163
	Alkalophilic *Bacillus* spp.		233
Dextranase	*B. megaterium*	Cell-bound enzymes catalyzing the exohydrolysis of dextran to glucose	355
	B. subtilis		355
Galactanase	*B. amyloliquefaciens*	Hydrolysis of the β-1,4-galactosidic linkages in soybean arabinogalactan	77
	B. subtilis var. *amylosachariticus*		79
β-1,3-glucanase	*B. circulans*	Endohydrolysis of the β-1,3-glycosidic linkages in laminarin and related glucans	167, 263
	B. polymyxa		75
	B. subtilis		86,197
	Alkalophilic *Bacillus* sp.		128
β-1,6-glucanase	*B. circulans*	Hydrolysis of pustulan and related glucans	262
Isoamylase	*B. amyloliquefaciens*	Hydrolysis of the α-1,6-glycosidic branch linkages in glycogen, amylopectin, etc.	322, 323
	B. polymyxa		104
Levansucrase	*B. subtilis*		95
Lichenase	*B. pumilus*	Hydrolysis of the β-1,4-glucosidic linkages of lichenenan	305
Maltase	*B. subtilis*	Hydrolysis of the α-1,4 linkage of maltose and maltotriose	327
Mannanase	*B. amyloliquefaciens*	Endohydrolysis of the β-1,4-mannosidic linkages of mannan	78
Pectate lyase	*B. circulans*	Endocleavage of polygalacturonic acid by an eliminative reaction	144
	B. polymyxa		230, 231
	B. pumilus		60, 61
	B. sphaericus		209
	B. stearothermophilus		149
	B. subtilis		171, 328
	Alkalophilic *Bacillus* spp.		127, 157

TABLE 1—*Continued*

Enzyme	Species	Comments	Reference
Phosphomannase	*B. circulans*	Cleaves phosphomannan from yeast cell walls	207, 208
Pullulanase	Alkalophilic *Bacillus* sp.	Endohydrolysis of the α-1,6 linkage of pullulan	234
Xylanase	*B. amyloliquefaciens*	Hydrolysis of xylans; the specificity of the enzymes has not been studied in detail	78
	B. firmus		86
	B. polymyxa		86
	B. subtilus		86
	B. subtilis var. *amylosaccharíticus*		79
Proteases			
Alkalophilic protease	Alkalophilic *Bacillus* spp.	Serine enzymes from alkalophilic species with very high pH optima	6, 125 317
Aminopeptidase	*B. licheniformis*		108
	B. subtilis		252, 326
Esterase	*B. subtilis*	Serine enzyme with high esterolytic and low proteolytic activity	195
Halophilic protease	*Bacillus* sp.	Produced optimally in media containing 1.0 M NaCl	146
Metal protease	*B. amyloliquefaciens*	Enzymes require Ca^{2+} for stability and Zn^{2+} for activity; pH optimum at or near neutrality	155, 186
	B. cereus		81
	B. licheniformis		108
	B. megaterium		151, 218, 219
	B. polymyxa		85
	B. subtilis		216
	B. subtilis var. *amylosaccharíticus*		150, 320
	B. thermoproteolyticus		80, 186, 238
	B. thuringiensis		187
Serine protease	*B. amyloliquefaciens*	The subtilisins; alkaline pH optima, serine residue at or near the active site	152
	B. licheniformis		154
	B. pumilus		154
	B. subtilis		216
	B. subtilis var. *amylosaccharíticus*		150, 154
Serine-metal protease	*B. licheniformis*	Hybrid enzymes with characteristics of both the serine and metal proteases	325
	B. pumilus		41
Penicillinases			
β-Lactamase	*B. anthracis*	Hydrolysis of the amide bond in the β-lactam ring of penicillins and cephalosporins	86
	B. cereus		62, 63, 136
	B. licheniformis		210, 248
	B. megaterium		86
	B. subtilis		86
Penicillin amidase	*B. megaterium*	Hydrolysis of the peptide linkage of penicillin	1,42
Nucleases and phosphatases			
Alkaline phosphatase	*B. amyloliquefaciens*	Often cell bound, the enzyme is extracellular in these species	303
	B. cereus		39
	B. subtilis		39
	Alkalophilic *Bacillus* sp.		156
Deoxyribonuclease-ribonuclease	*B. amyloliquefaciens*	A large number of DNases, RNases and phosphodiesterases with individual properties have been purified	113, 264
	B. cereus		265
	B. pumilus		265
	B. subtilis		148, 160, 232
3-Nucleotidase	*B. subtilis*	Active on both ribo- and deoxyribonucleotides	294
5-Nucleotidase	*B. cereus*	Cell-bound enzyme in these species	39
	B. megaterium		
	B. subtilis	Also possesses nucleoside diphosphate-sugar hydrolase activity	203
	Bacillus spp.	Also possesses RNA phosphodiesterase activity	139

TABLE 1—*Continued*

Enzyme	Species	Comments	Reference
Bacteriolytic enzymes			
Endo-*N*-acetylglucosaminidase	*B. licheniformis*		33
	B. subtilis		87
Exo-*N*-acetylglucosaminidase	*B. subtilis*		12, 28, 242
Endo-*N*-acetylmuramidase	*B. subtilis*	True lysozyme	225, 239 309
Exo-*N*-acetylmuramidase	*B. subtilis*		65, 66
N-acetyl-muramyl-L-alanine amidase	*B. licheniformis*	A cell-bound enzyme; the major autolysin	33
	B. subtilis		
Lipase	*B. licheniformis*	Hydrolysis of triacylglycerol to diacylglycerol and a fatty acid anion	143
Phospholipase C	*B. anthracis*	Responsible for the "egg-yolk" reaction	109
	B. cereus		164, 293, 359
	B. thuringiensis		
Thiaminase	*B. thiaminolyticus*		72

Included in this group are the serine proteases from *B. subtilis* Marburg and *B. subtilis* SAC (150). The group A enzymes are less stable below pH 9.5 and have a higher esterase activity than the group B enzymes (150).

The metal proteases are a group of metalloendopeptidases that show maximal activity at or near neutrality. They are sensitive to metal-chelating agents and do not possess esterase activity. The enzyme from *B. subtilis* possesses 1 g-atom of zinc per molecular weight of 38,000 to 40,000, which is essential for activity, and Ca^{2+} has been shown to be important for enzyme stability (200). With the exception of thermolysin, the metal protease synthesized by the thermophile *Bacillus thermoproteolyticus* (80, 238), which retains 90% activity after 30 min of incubation at 70°C, these enzymes are the least stable of the *Bacillus* proteases.

B. megaterium (151, 219), *B. polymyxa* (155), *B. thermoproteolyticus* (155), and *B. thuringiensis* (187) secrete only the metal protease. The subtilisin Carlsberg-producing species, *B. licheniformis* and *B. pumilus*, secrete only this enzyme in quantity, and strains that simultaneously produce the metal enzyme do so in negligible amounts (150). The species that produce subtilisin Novo, *B. subtilis* and *B. amyloliquefaciens*, produce both this and the metal enzyme (150), and during sporulation, *B. subtilis* Marburg secretes a third protease, the esterase (216). Although Keay (150) has attempted to classify bacilli into groups on the basis of the types and combinations of proteases produced, it would seem that examination of a large spectrum of species by rigidly standardized conditions will be necessary before any conclusive results are obtained.

The term penicillinase is normally reserved for β-lactamase, the enzyme that hydrolyzes the amide bond in the β-lactam ring of 6-aminopenicillanic acid or 7-aminocephalosporanic acid and of their *N*-acyl derivatives (44). This enzyme has been reported to be produced by many *Bacillus* species (86), but the penicillinases from *B. cereus* and *B. licheniformis* have been studied in most detail.

B. cereus 569 produces two types of extracellular β-lactamase. These enzymes are coded for by different genes (unlike the situation in *B. licheniformis*) and no immunological affinities between them have been detected. β-Lactamase I, an enzyme with relatively little activity on many cephalosporins, displays many of the properties typical of the penicillinases from other gram-positive sources, e.g., *Staphylococcus aureus* (136, 192, 313). β-Lactamase II has a number of unusual chemical and enzymological properties that set it apart from other penicillinases. Like the enzymes from gram-negative sources, β-lactamase II has a high activity towards cephalosporin derivatives and a molecular weight of about 22,000 (172). However, unlike all the other penicillinases that have been studied, it is a metalloenzyme requiring Zn^{2+} for full activity (63). A third membrane-bound β-lactamase has been described in *B. cereus* 569 (45).

B. licheniformis 749 synthesizes a single extracellular penicillinase that can also be detected as a membrane-bound hydrophobic enzyme. This extracellular penicillinase is closely related to the β-lactamase I of *B. cereus*—the molecules cross-react serologically, and a comparison of their structures has revealed significant homology (2, 313).

Penicillin amidase (penicillin acylase, penicillin aminohydrolase) hydrolyzes the peptide linkage of benzylpenicillin to yield phenylacetic acid and 6-aminopenicillanic acid. The latter is used for the industrial synthesis of substituted penicillins. Penicillin amidase is not commonly produced by the bacilli (130), the enzyme from *B. megaterium* being the most extensively characterized (1, 42).

The most intensively studied nuclease of the genus is the extracellular RNase from *B. amyloliquefaciens*, Barnase (113). This enzyme has a molecular weight of 12,382, is insensitive to EDTA, and hydrolyzes ribonucleic acid (RNA) (but not deoxyribonucleic acid [DNA]) via cyclic terminal phosphate intermediates (264). The RNase from *B. pumilus* is related to Barnase in size and is similarly insensitive to EDTA (265). In contrast, the enzyme from *B. cereus* is larger, is inactivated by EDTA, and nonspecifically hydrolyzes RNA (but not DNA) to 3'-nucleotides (265). Of the four extracellular nucleases that have been purified from the culture fluid of *B. subtilis* SB19 (148), three catalyze the Ca^{2+}-dependent exonucleolytic hydrolysis of both RNA and DNA from the 5' end of the polynucleotide to nucleotide 3'-monophosphates. This property has been utilized for 5'-end-group analysis of DNA (147). The fourth enzyme hydrolyzes only single-stranded DNA and exonucleolytically produces 3'-monophosphates (148). The extracellular nucleases of *B. subtilis* Marburg have been resolved into five fractions by chromatography on diethylamino-

TABLE 2. *Descriptions of genetic loci involved in exoenzyme synthesis in B. subtilis*

Genetic locus	Phenotype and comments	References
amyE	Structural gene for α-amylase; maps on the origin side of *aroI* in the order *lin-amyE-aroI-narB*	342, 352, 353
amyR1	Regulatory gene for α-amylase; cotransforms with *amyE*; suggested order *lin-amyR-amyE-aroI*.	341, 342, 351
amyR2	Amy^h; regulatory gene for α-amylase from *B. subtilis* NAT; allelic to *amyR1*	347, 348
amyR3	Amy^h; regulatory gene for α-amylase from *B. subtilis* SAC; phenotypically identical to *amyR2*	
amyB	Amy^h Npr^h Sep^h Mot$^-$; map position and relation to other pleiotropic mutations unknown	288
amyC	Amy^h Prt$^-$	288
catA	Prt^h; hyperproduction of proteases in the presence of glucose	138
hpr	Npr^h Sep^h Amy$^+$; hyperproduction of neutral and serine proteases, normal α-amylase synthesis	120
lad	Prt^h, Spo$^-$; late production of protease, sporulation defective	10
nprE	Structural gene for neutral protease	321
nprR1	Regulatory gene for neutral protease from *B. subtilis* Marburg	321
nprR2	Npr^h; regulatory gene for neutral protease from *B. subtilis* NAT; alellic to *nprR1*	321
pap	Amy^h Npr^h Sep^h Mot$^-$ Tfm$^-$ Aln$^-$; exoenzyme hyperproducing mutant deficient in flagella, transformation, and autolysin; unlinked to *amyR*	8, 346, 347
sacB	Structural gene for LS; maps between *cysB3* and *hisA1*	183
sacR	Regulatory gene for LS	182
sacS	Regulatory gene for LS and intracellular sucrase; linked to *purA* and *sacA* by PBS1 transduction	182
sacU	Lvs^h Prt^h Csp$^-$ Amy$^+$ Mot$^\pm$; maps between *gtaA* and *uvr*	170, 182
sep	Structural gene for serine protease; relationship to sporulation uncertain.	179, 180, 221
tmr	Amy^h Tm^r; resistance to tunicamycin; associated with hyperproduction of α-amylase	276
penP	Structural gene for penicillinase in *B. licheniformis*; can be transduced to *B. subtilis* 168.	292
penI	Repressor gene for penicillinase in *B. licheniformis*	292
penR	Regulator gene for penicillinase in *B. licheniformis*.	292
Spo0a	Spo$^-$ Amy$^-$ Npr$^-$ Sep$^-$ Pleitropic sporulation mutants deranged in extracellular enzyme synthesis	137, 213
Spo0b	Spo$^-$ Amy$^+$ Est$^-$ Npr$^-$ Sep$^-$	
Spo0c	Spo$^-$ Est$^-$ Npr$^-$ Sep$^-$	
spoA	Spo$^-$ Amy$^+$ Npr$^-$ Sep$^-$ Spo0a and *spoA* are probably alleles of a single locus	122
spoB	Spo$^-$ Npr$^-$ Sep$^-$	122
spoE	Spo$^-$ Npr$^-$ Sep$^-$	122
spoF	Spo$^-$ Npr$^-$ Sep$^-$	122

ethyl (DEAE)-cellulose (232). Enzyme I hydrolyzed both RNA and DNA to 3'-mononucleotides, and a second enzyme was specific for RNA. It is probably related to the RNase from *B. cereus* or *B. pumilus* described above. An extracellular factor that induces the competent state for DNA-mediated transformation in *B. subtilis* 168 has been shown to have nucleolytic activity (7) although its relationship to the extracellular nucleases is unknown. The factor degrades one strand of double-stranded DNA into acid-soluble nucleotides.

Extracellular alkaline phosphatase has been reported in the culture medium of *B. subtilis* (38), *B. cereus* (39), *B. amyloliquefaciens* (303), and an alkalophilic *Bacillus* sp. (156) but does not commonly occur as an extracellular enzyme. In other strains of *B. subtilis* (91, 339) and *B. megaterium* (339), it is membrane associated and cannot be detected in the culture fluid.

Enzymic hydrolysis of the peptidoglycan of bacterial cell walls may occur in two major loci: (i) cleavage of the peptide cross-linking chains or (ii) cleavage of the polysaccharide backbone. The most prominent autolysin of both *B. subtilis* and *B. licheniformis* is the *N*-acetylmuramyl-L-alanine amidase, which belongs to the former group by virtue of its attack on the bond between *N*-acetylmuramic acid and the L-alanine residue of the peptide side chain (33). This enzyme is cell bound and associated with the teichoic acid component of the cell wall and is believed to be functional in cell wall turnover and growth. Enzymes responsible for the hydrolysis of the peptidoglycan backbone are well represented in the genus. Endo-β-*N*-acetylmuramidase (true lysozyme) hydrolyzes the β-1,4 linkage of *N*-acetylglucosaminyl-*N*-acetylmuramic acid. This enzyme is produced extracellularly by *B. subtilis* YT-25 (228) and *B. subtilis* K-77 (239). An exo-β-*N*-acetylmuramidase is secreted by *B. subtilis* B (65, 66). The enzyme has been characterized, and its ability to attack several synthetic and natural substrates has been assessed. From these studies it was concluded that the enzyme is specific for substrates with nonreducing *N*-acetylmuramic acid end groups (65).

The alternative linkage in the polysaccharide backbone of peptidoglycan is susceptible to hydrolysis by β-*N*-acetylglucosaminidases. These have been described in cultures of *B. subtilis* and *B. licheniformis* (12, 33, 65, 241).

PHYSIOLOGICAL FUNCTIONS

Most of the truly extracellular enzymes synthesized by bacilli can, from a teleological standpoint, be described as "scavenger enzymes." Many have no cellular substrate, and it would seem probable that their sole function is to degrade polymers in the environment and thus supply the bacterium with an assimilable source of nutrients. However, those exoenzymes that possess cellular substrates, e.g., proteases, cell wall lytic enzymes, nucleases, etc., have been implicated in a number of physiological processes including sporulation, cell wall turnover and growth, and DNA-mediated transformation.

Sporulation

The maximal synthesis of extracellular enzymes normally occurs before sporulation in the late exponential and early stationary phases of growth (280). In early studies this observation suggested the possibility of causality between exoenzyme synthesis and sporulation. However, the transition from exponential-phase to stationary-phase growth is often accompanied by a depletion of the carbon source from the medium, and the change in cultural conditions derepresses many catabolite-repressed genes. Consequently, it is difficult to decide whether the appearance of an enzyme is due to the altered environment or the expression of a sporulation-specific gene. In many instances the use of mutants has clarified this situation. Clearly, if a mutant is deficient in a particular enzyme and yet sporulates normally, this enzyme can play no part in sporulation. α-Amylase is such an enzyme, and amylase-defective (Amy^-) mutants of *B. subtilis* grow and sporulate normally (280), thus dissociating this enzyme from any function in sporulation. The converse, i.e., that asporogenous (Spo^-) mutants defective in a particular exoenzyme implies a causal relationship between the two events, is not necessarily true. Such a situation is more often the result of a pleiotropic mutation in a regulatory gene and does not infer causality. A direct relationship can only be certified if the *spo* mutation is shown to map in the structural gene of the enzyme concerned.

The biochemical events, including exoenzyme synthesis associated with sporulation, have been divided into three physiological categories (59, 196). These are (i) the primary sequence of dependent events specifically concerned with spore formation (the term "dependent" describes an event that will not occur if the sequence is broken); (ii) side events that are not specifically involved in the process but are initiated by the occurrence of some of the events in (i); and (iii) phenotypic changes induced by the changes in cultural conditions that initiate

sporulation. These events are not involved in the process of sporulation. The isolation and characterization of Spo⁻ and exoenzyme-deficient mutants and the use of specific inhibitors has helped to clarify the role of exoenzymes in sporulation by assigning them to the three categories described above.

Early studies of protease synthesis during the batch culture growth cycle established a relationship between the maximal rate of protease synthesis and the initiation of sporulation (13, 185). Furthermore, the analysis of Spo⁻ mutants revealed that many of these strains did not produce extracellular protease and that reversion to sporogeny was accompanied by the ability to synthesize protease (279). The early studies were ambiguous, however, because they failed to distinguish the different proteases that were being secreted by the bacilli. All mutants isolated to date that display altered serine protease activity also possess altered sporulation characteristics. In most instances, this is probably due to pleiotropic mutations, but, nevertheless, it has been accepted as strong evidence that the serine protease is functional in sporulation. Moreover, Leighton et al. (179, 180) described a temperature-sensitive (ts) mutant of *B. subtilis* that produced an inactive serine protease and was asporogenous at 47°C, the nonpermissive temperature. Revertants to wild type (frequency of approximately 10^{-7}) invariably acquired both a normal serine protease and the ability to sporulate at 47°C. The serine proteases from the parent and the mutant have been extensively characterized to confirm that the pleiotropic phenotype was the result of a lesion in the structural gene for serine protease (179). Millet et al. (221) have similarly isolated a ts mutant of *B. subtilis* that has a greatly reduced level of serine protease and esterase activity and is asporogenous with a phenotype similar to Spo0a mutants at the nonpermissive temperature, 42°C. Reversion of this mutant to Spo⁺ (frequency of approximately 10^{-8}) is invariably accompanied by the excretion of normal amounts of serine protease and esterase. However, Millet et al. (221; personal communication) have succeeded in dissociating the ts serine protease and ts Spo0 phenotypes in both these strains. Transformation of wild-type *B. subtilis* with DNA from either of the two mutants has yielded ts transformants that were asporogenous but retained normal serine protease activity at the nonpermissive temperatures. Thus, it would appear that an intact serine protease may not be an absolute requirement for sporulation to proceed.

Some evidence for the involvement of serine proteases in sporulation has been provided by inhibitor studies. Phenylmethylsulfonylfluoride (PMSF) inhibits extracellular serine protease activity and prevents sporulation in *B. subtilis* (58). However, the use of "specific" serine protease inhibitors such as PMSF for studies of proteolysis in *Escherichia coli* have recently been criticized. Schechter et al. (283) have shown that sulfonylfluorides cause a 95% drop in the adenosine triphosphate (ATP) levels of stationary-phase *E. coli* cells even though they have little effect on ATP levels in exponentially growing cells. Furthermore, with most inhibitor studies performed in vivo, it is difficult to be certain that the inhibitor is acting specifically. For example, *B. subtilis* synthesizes two PMSF-sensitive serine proteases (254)—one an intracellular enzyme and the other the exoenzyme. Finally, it is difficult to assign a particular function in sporulation to extracellular serine protease. It is unlikely that it would be required in its extracellular form, and although intracellular functions for extracellular serine protease have been proposed (71), our present understanding of the secretion of exoenzymes in bacilli precludes the possibility of it accumulating within the cell to fulfill these requirements.

An intracellular serine protease occurring exclusively during sporulation has recently been purified from *B. subtilis* (254). This enzyme differs markedly from the extracellular serine protease of this species, but it has many characteristics in common with the cytoplasmic serine protease synthesized during the sporulation of *B. megaterium* (217). A requirement for intracellular protease is indicated by the extensive protein turnover occurring during sporulation in most bacilli (71), and a mutant ts for the cytoplasmic serine protease is asporogenous and deficient in protein turnover at the nonpermissive temperature (306). Unfortunately, the reversion frequencies of this mutant indicate that the pleiotropic phenotype may be the result of two independent mutations.

The metal protease can be more easily dismissed from sporulation than the serine enzyme. Mutants of *B. subtilis* (107), *B. megaterium* (220), and *B. cereus* (3, 185), which sporulate normally but are completely deficient in neutral protease activity, have been isolated. This enzyme cannot therefore be functional in the process of sporulation in these bacilli. Dancer and Mandelstam (59) observed that thymine starvation, which prevents sporulation in *B. subtilis*, also prevented the synthesis of neutral protease, whereas it had no effect on the synthesis of α-amylase, an exoenzyme for

which Amy$^-$ Spo$^+$ mutants of *B. subtilis* have been described (279). On this basis they categorized the neutral protease as an enzyme not primarily involved in sporulation but whose synthesis is dependent on the sequence of events leading to the formation of the endospore (ii, above) and α-amylase as an enzyme completely divorced from sporulation but synthesized in response to the change in cultural conditions associated with the end of exponential growth.

The esterase, the third extracellular protease of *B. subtilis*, has not been studied in detail, and mutants solely deficient in this enzyme have not been isolated. The physiological role of the esterase remains unclear (195).

Cell wall lytic enzymes have been implicated in sporulation by Schaeffer (279), who isolated five Spo$^-$ mutants unable to produce lytic enzymes. Unfortunately, the situation is complicated by the multiplicity of these enzymes. Nevertheless, Ortiz (240) has described mutants of *B. subtilis* 168 that were deficient in exo-β-*N*-acetylglucosaminidase but grew and sporulated normally. It therefore seems probable that this enzyme, like α-amylase, has a purely "scavenger" function in *B. subtilis*.

The relationship between the total extracellular RNase activity and sporulation of *B. subtilis* has been studied by mutant isolation (279). Of seven mutants with altered RNase synthesis, one synthesized the enzyme(s) late and sporulated late, four produced reduced amounts of RNase and were oligosporogenous, and two asporogenous mutants were completely deficient in extracellular RNase activity. These studies indicate that at least partial RNase activity may be required for sporulation, but the individual enzymes (*B. subtilis* produces at least four nucleolytic enzymes [148]) will have to be examined before any conclusive results can be obtained. Thymine starvation coordinately inhibits total RNase secretion and sporulation in *B. subtilis* (59), suggesting that the enzyme(s), like the proteases, belongs to category (ii).

Cell Wall Metabolism

A number of physiological functions have been proposed for the autolysins including cell wall growth, peptidoglycan turnover, cell separation, and DNA-mediated transformation. The evidence for these roles has been summarized in recent reviews (93, 121). The most prominent autolysin is the cell-bound *N*-acetylmuramyl-L-alanine amidase (33). Mutants defective in this enzyme have been connected with morphological aberrations such as chain formation or change in shape (Rod$^-$ mutants). Brown et al. (32) recently studied two ts mutants that grew as rods at 30°C but as cocci at 45°C. The rods contained about 30 times more amidase than the cocci and the cell walls contained proportionally more teichoic acid. Unfortunately, the data did not demonstrate that amidase deficiency was a causal factor in the morphological change. The simplest explanation of the data was that the pleiotropic effects were due to a lesion in a regulatory gene controlling several functions related to the cell surface.

The exo-β-*N*-acetylmuramidase and exo-β-*N*-acetylglucosaminidase of *B. subtilis* B are complementary in activity. Del Río and Berkeley (65) suggest that they are involved in cell wall metabolism rather than just scavenging because of their high degree of substrate specificity. But the kinetics of their production (maximal synthesis occurs late in exponential-phase growth) and the ability of a deficient mutant to grow normally (240, 241) exclude an involvement in any essential growth process. A possible function suggested by Del Río and Berkeley (65) is the degradation of the wall at the onset of some growth limitation to supply an oxidizable substrate and/or material for biosynthesis. The combined activity of the amidase, the exo-β-*N*-acetylmuramidase, and the exo-β-*N*-acetylglucosaminidase could readily convert the peptidoglycan to oxidizable monosaccharides when the cell is starved.

Transformation

Transformation only occurs after the receptor cells have developed the capacity to irreversibly bind DNA (competence; for review see references 235, 350). Some nontransformable mutants display a reduction in autolytic activity, but this correlation has not been entirely consistent (350). A competence-inducing factor in culture supernatant fluids and extracts of *B. subtilis* that stimulates transformation in a fashion similar to that in streptococcus and pneumococcus has been identified. This factor has been purified and shown to possess both cell wall-lytic and DNase activity, although the exact nature of the former has not yet been determined (7). The observations that a low concentration of lysozyme stimulates transformation (315) and that the binding of DNA may activate autolysin (301) support the concept of a role for cell wall-lytic enzymes in transformation.

When DNA is exposed to either competent or noncompetent *B. subtilis* cells the transforming ability of the unadsorbed DNA decreases (115, 142) due to the introduction of double-strand scissons (142). In addition, competent cells pro-

duce an extensive breakdown of the DNA to acid-soluble products. This cell-bound exonucleolytic activity can be detected in competent cells only and is inhibited by EDTA. Transformation is similarly inhibited by EDTA, suggesting that the exonucleolytic activity is involved in the process. DNA molecules that bind to competent cells in the presence of EDTA remain susceptible to externally added DNase I (225), indicating that the conversion of bound DNA from a DNase-sensitive to -resistant form is dependent on metal ions (246), and evidence suggests that it involves degradation of the double-stranded DNA into a single-stranded intermediate. In support of this, Chilton and Hall (43) and Tevethia and Mandel (312) have shown that EDTA blocks transformation by native, but not by single-stranded, DNA. It is possible that the exonucleolytic activity associated with competent cells (142) converts the double-stranded to single-stranded DNA during binding. Similarly, the extracellular competence-inducing factor described by Ayad and Shimmin (7) also possesses this ability and the two enzymes may perhaps be the same. However, further characterization of the cell-bound and extracellular nucleases is required before specific functions can be assigned to these enzymes.

REGULATION OF SYNTHESIS

Batch Culture

A brief examination of the extensive literature concerned with the stage during the batch culture growth cycle at which exoenzyme synthesis becomes maximal will reveal a great deal of confusion. In most examples there is little or no exoenzyme secretion during the lag or exponential-growth phases but, as the mean generation time of the culture increases, synthesis is initiated and becomes maximal during the early stationary phase. Confusion has arisen over this relatively straightforward pattern of synthesis for several reasons. First, a large variety of often poorly classified and misnamed strains and species have been used for these studies. In many instances these organisms are of industrial origin and have been extensively mutated to obtain high yields of exoenzyme. They have consequently lost their normal metabolic controls and, from the viewpoint of exoenzyme synthesis, bear little resemblance to the wild-type strain from which they originate. This situation is further aggravated by the use of media of widely differing complexity often containing commercial nutrient sources of varying and unknown composition. In these conditions, exponential growth is often followed by a lengthy period of deceleration, often extending from 12 to 36 h, during which the bacteria gradually enter stationary phase and ultimately sporulate. The metabolic activity and stable RNA content of these cells will be radically different from those grown in minimal medium containing a single carbon source and in which the transition from exponential to stationary phase is rapid (118). Variations in exoenzyme synthesis are therefore to be expected from bacteria grown in such different environments. Finally, most groups working in this field develop their own enzyme assay systems, each with its own units of activity. This situation enormously complicates the already difficult problem of correlating different studies. Nevertheless, from this scene of confusion two definite patterns of exoenzyme synthesis during the growth cycle emerge: (i) a very low rate of secretion during active growth followed by increased synthesis in the late exponential and early stationary phases of growth and (ii) increase in the synthesis and secretion of the exoenzyme accompanying growth and decreasing as the culture enters stationary phase.

The kinetics of α-amylase production by *B. subtilis* Marburg growing in complex medium adhere to the former pattern, and maximal synthesis occurs after growth has ceased (287, 346). However, *B. subtilis* W23 grown in minimal medium containing starch as the main carbon source synthesizes α-amylase at a low and linear rate throughout the growth cycle (333). Of the several possible explanations for this anomaly, the most plausible relates to the different media. The minimal salts-starch medium supported a lower growth rate and reduced final cell density compared with nutrient broth. This would have the effect of "flattening" the growth curve and consequently minimizing any variation in the rate of α-amylase synthesis. *B. licheniformis* (269), *B. macerans* (177), and the highly amylolytic bacilli, *B. amyloliquefaciens*, *B. subtilis* SAC, and *B. subtilis* NAT (46, 348) synthesize amylase maximally during the latter stages of the growth cycle. Thus amylase synthesis among these bacilli differs primarily in magnitude rather than the pattern of secretion throughout the growth cycle.

The three proteases of *B. subtilis* Marburg are virtually undetectable during logarithmic growth in nutrient broth (216) or glutamate-citrate (249) medium. The enzymes are secreted coordinately between the end of logarithmic growth (t_o) and the appearence of the prespore (t_4–t_5). The ratio of neutral:serine protease has been analyzed during this period, and the results suggest that synthesis of the former slightly precedes that of the serine protease (321). *B. amyloliquefaciens*, *B. subtilis* SAC,

and *B. subtilis* NAT produce a high proteolytic activity in the medium, which is principally due to the neutral enzyme (23, 321), although the ratio of neutral:serine protease is partially dependent on the composition of the growth medium (153). Protease synthesis by these species (46, 321), *B. pumilus*, and *B. licheniformis* (13, 153) occurs predominantly in the stationary phase and parallels α-amylase synthesis. The synthesis of neutral protease by *B. megaterium* (220) is highly dependent on the cultural conditions. In minimal medium the enzyme is synthesized throughout the growth cycle, but in complex medium it is repressed during growth and only synthesized before sporulation. Neutral protease synthesis by *B. cereus* (185) and *B. thuringiensis* (187) has been examined only with complex growth media in which the enzyme is similarly repressed until growth ceases. Other exoenzymes synthesized after exponential growth include the nucleases (148) and cell wall-lytic (28, 65) enzymes of *B. subtilis*, the endopolygalacturonic acid *trans*-eliminase of *B. pumilus* (61), and hemicellulases of *B. subtilis* SAC (79).

Exoenzyme synthesis in *B. polymyxa* does not conform to the general pattern. Laminarinase (75), protease (83), amylase (103), and xylanase (83) are synthesized during growth, and the rate of synthesis declines as the culture enters stationary phase. This may be a reflection of the media used in these studies. Laminarin, starch, and xylan were supplied as major carbon sources for examination of the respective enzymes, and the relatively slow growth rates achieved on these substrates will distort the exponential/stationary-phase growth pattern. Alternatively, it may be the result of variation in the control mechanisms operating in this species. Other examples of this pattern of synthesis include the phospholipase C of *B. cereus* (164), the pectinase (171) of a *B. subtilis* strain isolated from Sitka spruce, and the maltase of *B. subtilis* (327).

At least two exoenzymes are synthesized in response to a specific inducer molecule and, in the absence of this inducer, the basal activity is barely detectable. Perhaps coincidentally, the substrates for both of these enzymes, penicillinase and levansucrase (LS), are small molecules. Constitutive mutants of both penicillinase- and LS-producing strains have been isolated in which the enzymes are synthesized throughout the growth cycle.

Continuous Culture

The possibility of industrial enzyme production using large-scale continuous culture with the decided investment advantages of such a system has stimulated several studies in this area (35, 82, 319). The rate of postlogarithmic phase α-amylase synthesis by both *B. amyloliquefaciens* (116) and *B. subtilis* A32 (319) decreases rapidly as soon as fresh medium is added to the culture and growth is resumed. Even after prolonged addition of medium, steady-state synthesis of α-amylase by *B. amyloliquefaciens* cannot be achieved; the level fluctuates and ultimately declines (116). Synthesis of α-amylase by *B. subtilis* A32, when growing continuously, reached a steady-state after 80 h. The enzyme activity was 20% that of a comparable batch culture (319). Cyclodextrin glucanotransferase (CGT) synthesis by *B. macerans* growing in continuous culture has been studied by Lane and Pirt (177). At low dilution rates the rate of synthesis of the enzyme was highest, but at a fast growth rate CGT secretion was barely detectable. This inverse relationship between extracellular enzyme synthesis and growth rate has been similarly described for α-amylase synthesis by *B. licheniformis* (211) and is to be expected for products that are synthesized principally during the stationary phase.

The differences in the physiological state of bacteria growing in batch and continuous culture can be, at least partially, resolved by the use of multistage continuous systems (319). The multistage chemostat consists of a growth vessel into which medium is entering and from which culture is fed into a second vessel. The transferred culture is, to a certain extent, "shifted down," and a part of the growth curve after the exponential phase is adopted. The growth rate of the culture in the second or subsequent stages may be modified by the addition of fresh nutrient. Fencl et al. (82) arranged a two-stage chemostat culture of *B. subtilis* for the production of α-amylase. Optimal results were obtained when the growth rate in the first stage was high and in the second stage relatively low. This system produced better enzyme levels in the second stage than in a single-stage fermentor, but the activity was still 40% lower than that of equivalent batch cultures. Nevertheless, optimization of multistage continuous fermentors and the use of heterogeneous continuous fermentors may provide a valuable economic alternative to the batch culture production of exoenzymes.

Two important questions emerge from this outline of exoenzyme synthesis by bacilli during batch or continuous culture: first, what the mechanism is by which exoenzyme secretion is repressed until exponential-phase growth is virtually completed; and second, what control

process is responsible for the greater rate of exoenzyme synthesis in the highly amylolytic bacilli *B. amyloliquefaciens*, *B. subtilis* SAC, and *B. subtilis* NAT compared with *B. subtilis* Marburg. In all the examples examined so far, the process of exoenzyme secretion involves de novo protein synthesis and not precursor activation since inhibitors of protein synthesis prevent exoenzyme accumulation. Thus, the answers to these questions will lie in the mechanisms of regulation of protein synthesis in these organisms.

Catabolite Repression

Catabolite repression is the permanent repression of inducible or constitutive enzyme synthesis that occurs in the presence of glucose or some other rapidly metabolized carbon source. It provides an attractive explanation of many aspects of exoenzyme synthesis (280), and there is no doubt that it plays at least a contributory role in exoenzyme regulation. Because the cultural conditions that promote sporulation in bacilli (release from catabolite repression; 282) also derepress exoenzyme synthesis, many of the proposed mechanisms for triggering the initiation of sporulation can be applied with equal validity to the onset of the maximal rate of exoenzyme synthesis. The degree of involvement of exoenzymes in the process of sporulation can be classified at three levels: those that are intimately involved; those that are inessential, but nevertheless their synthesis is dependent on the expression of sporulation genes; and finally those that have been dissociated from sporulation but whose synthesis is triggered by the same cultural conditions that derepress sporulation genes (59; and see above). The catabolite repression scheme proposes that the release from repression that occurs at the end of exponential growth triggers the maximal rate of synthesis of all three categories of exoenzyme as it does the onset of sporulation. However, for the synthesis of those exoenzymes that are involved in, or dependent on, the expression of sporulation genes, the additional controls, e.g., RNA polymerase modification, which are necessary for sporulation to proceed, must be invoked.

The evidence in favor of catabolite repression as the principal control governing the expression of exoenzyme genes is best evaluated in relation to catabolite repression in *E. coli* in which the mechanisms are well understood. After induction or in constitutive mutants of *E. coli*, the rate of β-galactosidase synthesis is inversely proportional to the growth rate of the culture. Thus, the rate of synthesis is low in cells growing rapidly on glucose and galactose and higher in cells growing relatively slowly on lactate or succinate (226). Similarly, the rate of α-amylase synthesis in *B. subtilis* 168 is dependent on the carbon source in the order lactate > glutamate > maltose > glycerol > glucose, with glucose providing the fastest growth but lowest rate of α-amylase synthesis (287). Starch, glycerol, and glucose support doubling times for *B. amyloliquefaciens* of 2.2, 1.7, and 1.6 h, respectively, and the rate of α-amylase synthesis is inversely related (51) as it is in *B. stearothermophilus* (330) growing on similar carbon sources. Carbon-limited continuous cultures of *B. licheniformis* with either glucose, glutamate, or alanine as carbon source produce high levels of α-amylase; in contrast, nitrogen-limited cultures contain excess glucose and a barely detectable level of enzyme activity (211). Furthermore, under carbon-limited conditions, the amount of α-amylase produced by *B. licheniformis* in continuous culture is inversely proportional to the growth rate (211), and α-amylase synthesis by *B. amyloliquefaciens* in continuous culture follows essentially the same pattern (116). Returning to β-galactosidase synthesis in *E. coli*, a severe transient repression is observed when glucose is added to a constitutive or fully-induced culture growing on a nonrepressing carbon source; thereafter, the normal glucose-repressed rate of synthesis is adopted. The addition of glucose to *B. subtilis* cells synthesizing α-amylase results in a similar repression (251).

α-Amylase is not the only exoenzyme susceptible to catabolite repression. Protease synthesis in *B. licheniformis* (173), *B. subtilis* (71), and *B. amyloliquefaciens* (116) is repressed by glucose, as is the exo-β-*N*-acetylglucosaminidase of *B. subtilis* B (241). Frequently, studies of the nutritional factors that affect exoenzyme accumulation in cultures of various bacilli indicate that catabolite repression is not a general phenomenon and that high levels of exoenzyme are produced when glucose is the principal carbon source. Caution must be exercised in the interpretation of these results because the utilization of the glucose is seldom followed, and it may well be exhausted as the culture enters stationary phase, and, with the repression lifted, rapid synthesis of exoenzyme may be permitted. Nevertheless, there are exceptions; in particular a strain of *B. subtilis* has been isolated that synthesizes pectinase maximally when glucose is supplied as the carbon source (171).

The observations above lead to the conclusion, first proposed by Schaeffer (280), that the initiation of sporulation and many of the associated events including the switch from a mini-

mal to maximal rate of exoenzyme synthesis are regulated by catabolite-type repression.

Cyclic nucelotides. In gram-negative bacteria, particularly *E. coli*, glucose-induced catabolite and transient repression are effected by adenosine 3',5'-cyclic monophosphate (cyclic AMP; 259). This molecule exerts its influence through a protein, the catabolite gene activator protein (CAP) or cyclic AMP receptor protein (CRP). The cyclic AMP-CRP complex facilitates binding of RNA polymerase to the promoter regions of catabolite-sensitive operons (259). The intracellular concentration of cyclic AMP varies with growth or, more precisely, with the energetic state of the cell (259). It is some 20 times higher in cells growing slowly on proline, a poor source of carbon, than those growing rapidly on glucose (34). Furthermore, the transient repression of β-galactosidase synthesis due to the addition of glucose to an induced culture is accompanied by a rapid decrease of the intracellular cyclic AMP concentration (329) probably due to energy-dependent excretion (267). Much of the data implicating cyclic AMP in the mechanism of catabolite repression in *E. coli* have been summarized by Wayne and Rosen (329) and will not be repeated here. However, it should be emphasized that recent evidence has indicated that the modulation of cyclic AMP levels alone is insufficient to explain the range of catabolite-repression phenomena observed (270, 329). It has been suggested that guanosine cyclic 3':5'-monophosphate (cyclic GMP) is involved in the transcriptional control of catabolite-sensitive operons by the following observations: (i) the intracellular concentration of cyclic GMP in *E. coli* increases under catabolite-repressing conditions (15); (ii) a mutant CRP that appears to be activated by cyclic GMP in vivo has been isolated (270); (iii) cyclic GMP inhibits the expression of catabolite-sensitive genes in vivo (4) and in vitro (270). Although it has not been demonstrated in vivo that cyclic GMP is directly involved as a protagonist of cyclic AMP, the evidence is certainly suggestive.

If the regulation of metabolism in bacilli is analogous to that in *E. coli* a simple model can be postulated to explain the observed pattern of exoenzyme synthesis in these bacteria. Assuming exoenzyme operons to be constitutive (or fully induced) and catabolite sensitive, their expression would be very low during rapid growth due to the low intracellular concentration of cyclic AMP. As the culture enters stationary phase, the concentration of cyclic AMP increases, which, in conjunction with CRP, promotes the binding of RNA polymerase to catabolite-sensitive promoter regions and derepresses the transcription of exoenzyme messenger RNA (mRNA). Attractive as this hypothesis is, there is a major drawback. Cyclic AMP is not the universal regulator molecule it was once thought to be, and it cannot be detected in the several bacilli examined to date including: *B. subtilis* (110), *B. cereus* (291), *B. megaterium* (291), and *B. licheniformis* (15). In addition, adenyl cyclase and cyclic AMP phosphodiesterase, the enzymes responsible for the synthesis and degradation of cyclic AMP, are absent from *B. lichenformis* (15), *B. cereus* (134), and *B. subtilis* (134), and attempts to obtain adenyl cyclase-deficient or CRP-deficient mutants of *B. subtilis* have been unsuccessful (53). Cyclic AMP enters the *Bacillus* cell by a process of passive diffusion (11) but fails to relieve glucose repression of α-amylase synthesis in *B. subtilis* (251) and *B. licheniformis* (269). Claims that cyclic AMP increases the rate of α-amylase synthesis in bacilli (269, 354) must be treated with caution because of the increased reaction rate of α-amylase when assayed in the presence of cyclic AMP (251). The inability of cyclic AMP to overcome diauxie (338) or catabolite repression of sporulation in many bacilli (24) substantiates the evidence that cyclic AMP is not a regulatory molecule in most, if not all, *Bacillus* species.

However, Bernlohr et al. (15) have detected cyclic GMP in *B. licheniformis*. The levels are about one-fifth of those found in *E. coli* grown under similar conditions and at comparable rates. The maximum pool size is in the range 6 to 8 nM and decreases during exponential growth on glucose to a minimum level of less than 1 nM. It remains at this reduced level during late exponential growth and early stationary phase. It then increases to levels typical of early exponential-phase cells at a period when "commitment" to sporulation normally occurs. The size of the cyclic GMP pool appears to be proportional to the growth rate attained with different nutrients. The growth rate is low on glutamate as the sole carbon source (generation time about 3 h), and the cyclic GMP pool is low (<2 nM) compared with cells growing more rapidly on glucose in which the cyclic GMP pool is about 6 nM. The addition of glucose to a culture growing on glutamate increases the cyclic GMP pool threefold to a level typical of glucose-grown cells (15). The inhibitory effect of cyclic GMP on the transcription of β-galactosidase mRNA (76, 356) may also apply to catabolite-sensitive operons in *B. licheniformis*. If this is the case, then the modulation of cyclic GMP levels described above would explain the

low rate of exoenzyme synthesis observed in exponential-phase cultures, the inhibitory effect of glucose, and the increased rate of synthesis found in late exponential-phase and early stationary-phase cultures before sporulation. In the absence of an in vitro transcription system for *Bacillus* DNA, further clarification of the role of cyclic GMP in these bacteria must come from in vivo studies of the nucleotide and from the isolation and phenotypic examination of guanyl cyclase and cyclic GMP phosphodiesterase mutants.

Highly phosphorylated nucleotides. In a search for small molecules that may be involved in the initiation of sporulation and the process of catabolite repression, Rhaese et al. (255) chromatographed formic acid extracts of *B. subtilis* cells harvested at different periods in the growth cycle and have identified four unusual, highly phosphorylated nucleotides (HPN). These compounds, HPN I, II, III, and IV, have since been identified as adenosine tetraphosphate (ppApp), adenosine pentaphosphate (pppApp), a nucleotide with a tentative structure of ppZpUp where Z is an undetermined sugar, and adenosine hexaphosphate (pppAppp), respectively (257). The appearance of the nucleotides consistently correlates with the period of maximal exoenzyme synthesis and the initiation of sporulation associated with catabolite derepression. As *B. subtilis* enters stationary phase, HPN III accumulates intracellularly and HPN IV accumulates in the medium (256). If sporulation and exoenzyme synthesis are delayed by the addition of a high concentration of glucose to the medium, there is a similar delay in HPN synthesis and accumulation (257). In fact, in a range of growth conditions examined, HPN III and HPN IV synthesis occurred only when the glucose in the medium was exhausted. Furthermore, the premature induction of sporulation resulted in the premature synthesis of these HPN. This consistent correlation of HPN II and IV synthesis with sporulation and non-catabolite-repressing conditions strongly suggests that these nucleotides are involved in the regulation of gene expression and may be responsible for the induction of maximal exoenzyme synthesis in late exponential-phase cells.

HPN I and II can only be measured in vivo after the rigorous deprivation of glucose from the medium (256). Ribosomes from stationary-phase-sporulating *B. subtilis* cells synthesize HPN I and II in vitro (258). It is thought that these may be short-lived precursors in the synthesis of HPN IV, and their synthesis by sporulation-phase ribosomes is perhaps possible only because the conversion of these nucleotides into the hexaphosphate of adenosine in an in vitro system does not occur. Ribosomes from exponential-phase or glucose-repressed stationary-phase cells do not synthesize HPN I and II but instead synthesize the "magic spot" nucleotides guanosine tetraphosphate (ppGpp; MS1) and guanosine pentaphosphate (pppGpp; MS2).

The observation that HPN IV is excreted into the culture medium during stationary-phase growth (255) may explain the results of Saito and Yamamoto (269). These authors suspended young exponential-phase cultures of *B. licheniformis* in cell-free culture fluid. It was found that broth from 48-h cultures maximally derepressed α-amylase synthesis in the young cells, suggesting that an inducer accumulates extracellularly during growth. It may be that this hypothetical inducer is HPN IV. The addition of HPN II to cultures of *B. subtilis*, *B. amyloliquefaciens*, and *B. megaterium* stimulates sporulation and inhibits germination (227), but the effect on exoenzyme synthesis and other catabolite-sensitive functions has not been determined. Unfortunately, the effects of exogenous pppApp are not specific and ppGpp and pppGpp produce a similar response. This is somewhat surprising in view of the inability of Schaeffer et al. (281) to correlate ppGpp and pppGpp levels with sporogenesis. Furthermore, relaxed strains of *B. subtilis* unable to synthesize ppGpp and pppGpp sporulate normally, thus dissociating these molecules from a regulatory function in sporulation (257).

Control of Transcription

In the preceding section, I have discussed the regulation of exoenzyme synthesis by catabolite repression but omitted any reference to the stage during protein synthesis at which this may act. Catabolite repression is generally believed to control the rate of transcription of sensitive operons, and the following section reviews the evidence for such a system in extracellular enzyme synthesis in the bacilli. Experimental proof for the transcriptional control of an enzyme requires the demonstration, preferably directly, of the modulation of specific mRNA levels upon induction or derepression of an operon. In the absence of high-frequency specialized transducing phages for bacilli, the DNA from which could be used for the assay of specific RNA molecules by hybridization, more circuitous methods of mRNA estimation have been adopted. Only mRNA hybridizes with complementary DNA at a ratio of DNA to RNA of 10:1; the ratio must be increaed to 200:1 before extensive interference by ribosomal RNA

(rRNA) hybridization occurs. Brown and Coleman (31) have used this procedure to assay the mRNA content of *B. amyloliquefaciens* throughout the growth cycle. During exponential growth, mRNA accounted for a constant proportion (about 3%) of the total RNA in the cell. As the growth rate diminished, exoenzyme synthesis became maximal, and the mRNA content rose to approximately twice that of exponential-phase cells. This newly-synthesized mRNA was resolved into exoprotein or cellular protein fractions by measuring the rate of incorporation of labeled valine into extracellular and cellular proteins (assuming that both mRNA species are translated with equal efficiency). The results indicated that the increase in total mRNA observed in late exponential-phase cultures is due almost entirely to a 25-fold increase in exoprotein mRNA synthesis (31). This pattern of events has been compared with the synthesis of mRNA in *B. subtilis* in which a postexponential-phase increase in total mRNA was not observed, presumably because the technique is insufficiently sensitive to detect the small variations in mRNA that would be associated with the low level of exoenzyme synthesis in this organism.

The most compelling argument for the accuracy of these findings is that they comply with the expected results. However, there is a great deal of evidence to suggest that translational control mechanisms are operative during sporulation in *B. subtilis* (24), and the assumption that exocellular and cellular mRNA's are translated with equal efficiency may not be entirely valid. Nevertheless, the twofold increase in the total mRNA content in postexponential-phase *B. amyloliquefaciens* and the inability to detect this in *B. subtilis* provides good evidence for a transcriptional control over the expression of exoenzyme genes.

RNA polymerase modification. A model based on growth-associated changes in the transcriptional specificity of RNA polymerase has recently been proposed for the control of exoenzyme synthesis in bacilli (49). It is suggested that exoprotein mRNA synthesis is negligible during exponential growth because of the low affinity of RNA polymerase for initiation sites on the exoprotein operons. At the end of exponential growth a nutritional limitation is incurred. This inhibits rRNA synthesis with an accompanying reduction in cell protein mRNA formation. The net result is that more core RNA polymerase is available to combine with exoprotein-specific sigma factor, thus causing increased exoprotein mRNA synthesis and, hence, exoprotein itself (49).

Evidence in support of such a switch in the transcriptional specificity of RNA polymerase during the growth cycle has been obtained from studies of the incorporation of labeled uracil into rRNA during growth and sporulation. rRNA synthesis in *B. subtilis* declines at t_0 to virtually zero (131) or to a reduced level, depending on the nature of the medium (22). This cessation of rRNA synthesis is associated with two specific molecular events: a change in RNA polymerase specificity and a modification of the enzyme. The modification of RNA polymerase during the process of spore formation has been recently reviewed (193) but will be briefly reiterated here because of its proposed relevance to the regulation of exoenzyme synthesis.

The composition of RNA polymerase from vegetative *B. subtilis* cells largely depends on the method of purification. Purification by phosphocellulose chromatography yields a core enzyme consisting of the polypeptides β' (molecular weight 15,000), β (molecular weight 150,000), α (molecular weight 41,000), and ω (molecular weight 11,000) in the stoichiometry $\beta' \beta\alpha_2\omega_{1-4}$ (193). If RNA polymerase is purified in the absence of phosphocellulose chromatography it contains an additional 55,000-dalton polypeptide termed the σ-polypeptide. In the presence of σ, RNA polymerase preferentially initiates transcription at rRNA cistrons (132), and it transcribes both polydeoxy(adenylate-thymidylate) [poly(dA-dT)] and phage ϕe DNA templates in vitro. Vegetative cells contain σ factor and support the growth of ϕe. Sporulating *B. subtilis* cells appear to contain a specific component that inhibits σ activity probably by interfering with the binding of σ to RNA polymerase (190, 285, 316). Although RNA polymerase from sporulating cells lacks σ activity, it may be isolated in association with a number of sporulation-specific polypeptides (190). It is not known whether these direct specific gene transcription, but their involvement in the regulation of gene expression seems highly probable. The modified RNA polymerase from sporulating cells will not transcribe rRNA cistrons or DNA from ϕe, and sporulating cells do not support the growth of ϕe. A mutant *rfr*10, selected as rifampin resistant, sporulates at only 5% of the wild-type frequency. This mutant continues to synthesize rRNA after the completion of exponential growth (131), and RNA polymerase isolated from stationary-phase cultures of *rfr*10 transcribes rRNA cistrons in vitro. This not only suggests a regulatory role for RNA polymerase in sporulation but also indicates that the mechanism for reducing rRNA synthesis in postexponential-

phase *B. subtilis* cells is not dependent on ppGpp or pppGpp, as it is during the stringent response of both *B. subtilis* and *E. coli*, but on RNA polymerase modification. This supports the findings of Rhaese et al. (257), that relaxed strains of *B. subtilis*, which lack the ability to synthesize ppGpp and pppGpp, sporulate normally.

If the mechanism of exoenzyme regulation proposed by Coleman et al. (49) is correct, the change in template specificity of the RNA polymerase associated with postexponential-phase growth is a prerequisite for the increased exoenzyme synthesis observed during this period. It follows that all mutants impaired in RNA polymerase modification should be similarly impaired in exoenzyme synthesis. The isolation of a mutant that fails to modify its RNA polymerase specificity but nevertheless secretes wild-type levels of exoenzyme is sufficient to dissociate these events. RNA polymerase has been isolated from stationary-phase cultures of several stage 0 (Spo0) sporulation mutants and examined for its ability to transcribe ϕe DNA in vitro (27). A mutation in any one of six loci resulting in the Spo0 phenotype prevents the change in RNA polymerase template specificity, and the enzyme isolated at $t_{4.5}$, which from wild-type cells would be unable to transcribe ϕe DNA, performs this operation efficiently. All the Spo0 mutants examined were either totally or partially deficient in extracellular serine protease and esterase synthesis. Mutants blocked after stage 0, i.e., stages II, III, and IV, produced a modified RNA polymerase and secreted normal quantities of serine protease and esterase. Sonensheim et al. (299) isolated a large number of rifampin and streptolydigin-resistant mutants of *B. subtilis*, many of which were blocked at stage 0. The authors concluded that only those strains that sporulated less well than their parents were deranged for protease synthesis.

The situation is a little more confused when extended to include α-amylase and neutral protease synthesis. Spo0a strains produce a very low level of serine protease and reduced amounts of both neutral protease and α-amylase. Spo0b and Spo0c mutants synthesize reduced amounts of serine protease and esterase but normal levels of neutral protease (213). Spo0a, -b, and -c mutants are all blocked in the modification of RNA polymerase and the enzyme from stationary-phase cells can be isolated in association with the vegetative σ factor (102). Consequently, the modification of RNA polymerase is not necessary for the increased synthesis of neutral protease in stationary-phase cultures of *B. subtilis*. Hoch and Mathews (123) have placed several stage 0 sporulation mutations in five loci on the *B. subtilis* chromosome. Four of the five classes (*ABE* and *F*) produce reduced levels of both the serine and metal proteases, but the fifth (*H*) has not been quantitatively examined in this respect (26). The relationship between these mutants and those employed by Michel and Millet (213) remains largely obscure, but the weight of genetic evidence suggests that Spo0a and *spoA* mutations are allelic, and phenotypically, *spoB* and $Spo0b_2$ act like alleles of a single locus (122). *spoA* mutants produce normal amounts of α-amylase, indicating that RNA polymerase modification is not necessary for the increased synthesis of this exoenzyme during stationary phase.

In conclusion, therefore, it appears probable that the change in RNA polymerase template specificity is necessary for serine protease synthesis in view of the fact that all the Spo0 mutants isolated to date fail to undergo RNA polymerase modification and are impaired in serine protease synthesis. This may well account for the close association of serine protease synthesis and sporulation observed by others (58, 71, 180, 279). On the other hand, neutral protease and α-amylase are synthesized normally in some Spo0 mutants in the absence of RNA polymerase modification. Thus, the loss of the vegetative σ factor activity that occurs at the end of exponential growth cannot be involved in the initiation of synthesis of these exoenzymes. This conclusion is supported by the observations quoted by Bott (24) that several events including exoenzyme synthesis may precede alterations in RNA polymerase specificity and that the synthesis of neutral protease precedes the synthesis of serine protease (321).

Nucleotide precursors. Coleman et al. (49) envisage exoenzyme synthesis in *B. amyloliquefaciens* to be regulated by two mechanisms that can be superimposed. The first is the modification of RNA polymerase specificity such that exoenzyme operons are recognized, and the second is an increase in the size of the RNA precursor pool causing an increase in the rate of transcription. It is suggested that the increased "nucleotide" pool in postexponential-phase bacteria would result from the turnover of nontranslatable RNA.

There is general agreement that the net synthesis of RNA in *B. subtilis* ceases at the end of exponential growth (9, 70). The intracellular level of RNA remains constant throughout most of the sporulation process decreasing late in spore formation (70), although Brown and

Coleman observed a 17% reduction in the total RNA content of *B. subtilis* 168 during the transition from exponential- to stationary-phase growth (31). However, although net synthesis stops at t_o, measurement of the incorporation of radioactive precursors into RNA indicates that it is undergoing a rapid intracellular turnover. The rate of turnover is constant during sporulation and reaches 20% per hour (9). The percent incorporation of labeled uracil into rRNA and ribosomal precursors decreases drastically in sporulating cells, suggesting that rRNA genes are not transcribed (131). The ratio of tRNA to rRNA of 0.2 in vegetative cells increases during sporulation to 0.36 (70), a result of the increasing degradation of ribosomes.

RNA metabolism in postexponential-phase *B. amyloliquefaciens* follows a similar pattern. The RNA content of the cells declines at t_o (31), leading to an inverse relationship between the increase in the amount of orcinol-reacting material extracted by ice-cold trichloroacetic acid ("nucleotide" pool) and the decline in stable RNA. Furthermore, when the stable RNA of exponential-phase cells is labeled with [^{14}C]uracil, 15% of the label is lost during early stationary phase and can be recovered from the "nucleotide" pool (304). During this period, the "nucleotide" pool increases in size fourfold from 0.4% of the bacterial dry weight in exponential-phase cells to 1.4% in stationary-phase cells. It was proposed that it is the degradation of rRNA and increase in nucleotide precursors that initiates the maximal rate of exoenzyme synthesis in *B. amyloliquefaciens* (49, 304). The "nucleotide" pool of *B. licheniformis* increases by only 75% during this period (181) possibly reflecting the relatively low rate of exoenzyme synthesis by this bacterium. There are no comparable figures for *B. subtilis*, but analysis of the ATP content of growing and sporulating cells reveals either a constant ATP level throughout the stationary phase when growing in nutrient sporulation medium (165) or a decline in ATP and energy charge on the depletion of glucose from minimal medium (133). This pattern contrasts the rise in ATP observed in *B. amyloliquefaciens* during this period (304) and may be responsible for the low rate of exoenzyme synthesis in *B. subtilis*.

This model of exoenzyme regulation is consistent with our present understanding of RNA metabolism in these bacteria. RNA breakdown in *B. subtilis*, and possibly in the closely related *B. amyloliquefaciens*, is subject to regulation by nucleoside triphosphates acting as powerful inhibitors of phosphodiesterase II (PDase II) and 5'-nucleotidase (290). At the nucleotide triphosphate concentration occurring during vegetative growth, both PDase II and 5'-nucleotidase would be inhibited and phosphodiesterase I (PDase I) would be active to produce 5'-mononucleotides. These would be recycled to nucleoside triphosphates through successive phosphorylations. During stationary phase, the abrupt fall in the nucleotide triphosphate concentration in *B. subtilis* (133) would relieve the inhibition of PDase II and allow the synthesis of 3'-mononucleotides and, subsequently, of free nucleosides. These products could be stored in the spore and be available for germination when a rapid RNA turnover occurs (290). In *B. amyloliquefaciens*, however, the high nucleotide triphosphate level in stationary-phase cells (304) would continue to inhibit PDase II until a later stage and thus allow the recycling of nucleotide monophosphates into exoenzyme mRNA.

The evidence for the regulation of exoenzyme synthesis in bacilli by the availability of nucleotide precursors is very much circumstantial. The correlation of the "nucleotide" pool size of exoenzyme-secreting bacteria with the rate of secretion (52) is consistent with such a model but does not necessarily advocate it. In such a system it is difficult to differentiate cause and effect, and it is equally feasible that an enlarged nucleotide pool is present in cells rapidly synthesizing exoenzyme because of the increased metabolism of mRNA in these cells. Consistent with this suggestion is the finding that the addition of glucose to a late exponential-phase *B. subtilis* culture growing with maltose as principal carbon source results in a reduction in the rate of α-amylase synthesis and an increased "nucleotide" pool (unpublished data). This is presumably the result of continued hydrolysis of mRNA and glucose-induced inhibition of catabolite-sensitive mRNA transcription. Thus, in this organism the nucleotide pool size may provide a coarse control on the rate of exoenzyme synthesis, but this is overridden by catabolite repression. Clearly the regulation of RNA metabolism and precursor pools must be studied in much greater depth before such a model for the regulation of exoenzyme synthesis can be accepted.

Regulation of Translation

Coupling of transcription and translation. Postlogarithmic-phase cells of *B. amyloliquefaciens* that have been washed and suspended in fresh medium continue to synthesize extracellular enzymes. Both et al. (23) have used this procedure to study the regulation of protease synthesis in this bacterium. Suspension of the

cells in the presence of either rifampin or actinomycin D inhibits the incorporation of radioactively labeled uracil or valine into cellular RNA and protein, respectively. However, protease activity continues to increase in the culture filtrate for over 60 min in the presence of either drug. The possibility that this is simply due to the release of preformed enzyme was excluded by the observation that chloramphenicol rapidly inhibits protease secretion (23) and, in addition, labeling studies confirmed that the rifampin-resistant protease synthesis required de novo protein synthesis (23). When the cells are suspended in medium containing a high concentration of amino acids (0.5% casein hydrolysate) the synthesis of protease follows a biphasic time course. There is an initial rapid production of enzyme lasting about 30 min (phase 1). This is followed by a period of about 50 min during which protease synthesis is very low, and finally there is a resumption of synthesis at a linear rate (phase 2). Phase 1 protease synthesis is resistant to both actinomycin D and rifampin but phase 2 synthesis is promptly inhibited by the addition of either drug.

To explain these findings, Both et al. (23) proposed that postexponential-phase cells contain sufficient preformed mRNA to enable protease synthesis to be maintained for an extended period of time in the absence of RNA synthesis. It is suggested that this mRNA is composed of many copies of a short-lived species rather than a few copies of a long-lived messenger because the rapid inhibition of enzyme synthesis observed when rifampin is added to phase 2 cells indicates that the mRNA has a normal short half-life. When the cells are suspended in the presence of casein hydrolysate (0.5%) it is proposed that transcription is repressed, but phase 1 protease synthesis continues as the translation of the preformed mRNA. In the absence of transcription, this mRNA pool becomes depleted and phase 1 synthesis declines—thus, the plateau period at the end of phase 1. However, on prolonged incubation, derepression is thought to occur and protease synthesis resumes (phase 2) as the expression of coupled transcription and translation. Because the mRNA pool has been depleted, phase 2 protease synthesis is rapidly inhibited by rifampin.

These studies have been extended to include α-amylase and RNase synthesis in *B. amyloliquefaciens* (96). Both of these enzymes are synthesized for long periods (30 to 40 min) by suspended postexponential-phase cells in the presence of rifampin. The existence of an mRNA pool in early stationary-phase cultures of *B. amyloliquefaciens* therefore appears to be a general phenomenon associated with exoenzyme synthesis. Qualitatively similar results have been obtained with *B. subtilis* 168 (289), *B. subtilis* KA63 (161), *B. subtilis* B20 (251) and *Psuedomonas maltophilia* (21).

The effect of rifampin on the metabolism of these bacteria is crucially important in the interpretation of the above results, and the use of this drug for the estimation of mRNA half-lives has been heavily criticized by Coote et al. (54) because of the nonspecific cytotoxic effects it has on *B. subtilis* cells. However, Brown and Coleman (30) have validated its use in *B. amyloliquefaciens* for the measurement of mRNA stability and shown that it can be employed with reasonable confidence at a concentration of 10 μg/ml for the specific inhibition of transcription in this organism. Nevertheless, to completely eliminate the ambiguity of inhibitor studies employing either rifampin or actinomycin D, Kinoshita et al. (161) prevented transcription in a uracil-negative mutant of *B. subtilis* KA63 by starving the culture of uracil. Starvation completely inhibited the incorporation of labeled adenine into RNA and the increase in alkaline phosphatase activity and cell growth, but α-amylase synthesis continued for a considerable period despite the lack of mRNA synthesis.

Brown and Coleman (29) have challenged the validity of the results of Both et al. (23) and argue against the presence of an mRNA pool in *B. amyloliquefaciens*. These authors have demonstrated that the concentration of rifampin (0.5 μg/ml) used by Both et al. (23) totally inhibits mRNA synthesis for cellular proteins but inhibits exoenzyme mRNA synthesis by only 50%. They suggest that this relative resistance of exoenzyme mRNA synthesis to rifampin would account for the longevity of protease synthesis in the presence of rifampin and dismisses the necessity to invoke a pool of mRNA. However, these findings do not explain the rapid and complete inhibition of phase 2 protease synthesis by rifampin, nor do they account for the supporting evidence that actinomycin D and uracil starvation (161) produce identical results to those obtained with rifampin.

As described above, the total mRNA content of *B. amyloliquefaciens* assayed by hybridization with DNA at a ratio of DNA to RNA of 10:1 doubles as the culture enters stationary phase (31). This increased mRNA pool is composed of equal amounts of exoprotein and cellular protein mRNA, reflecting a 25-fold increase in exo-

protein mRNA as the culture shifts from exponential- to stationary-phase growth. This is consistent with the concept of a pool of mRNA capable of sustaining exoenzyme synthesis in the absence of transcription. To demonstrate multiple gene copies in this mRNA pool, Brown and Coleman (30) employed a serial hybridization technique in which randomly labeled RNA was exhaustively hybridized with DNA at a ratio of DNA to RNA of 5:1. The serial hybridization curves for both exponential- and postexponential-phase rapidly labeled RNA from *B. amyloliquefaciens* was almost identical (30), indicating that multiple copies of a few mRNA species were not present in the postexponential-phase cells. However, these hybridization results must be interpreted with care. Under the conditions employed, nonspecific binding of the RNA to DNA is normally low. But when the amounts of RNA offered for binding are greatly in excess of the available DNA sites, which is possible if multiple gene copies are present, a marked accentuation in the nonspecific binding of the input RNA to both the DNA and support filter occurs (215). This could account for the similarity in the serial hybridization curves of exponential- and postexponential-phase RNA.

It must be emphasized that an mRNA pool is not considered a prerequisite for exoenzyme synthesis (289). Phase 2 protease synthesis in *B. amyloliquefaciens* is thought to be the result of coupled transcription and translation in the absence of an mRNA pool. Furthermore, if an exponential-phase culture of *B. amyloliquefaciens* is concentrated fourfold, it enters stationary phase prematurely. This inhibition of growth is accompanied by an immediate initiation of protease synthesis, suggesting that exoenzyme synthesis can occur before a pool of mRNA would have had sufficient time to accumulate (29).

mRNA stability. The characteristics of mRNA synthesis and degradation in *B. subtilis* appear to conform to the procaryotic pattern. In exponential-phase cultures of *B. subtilis* 168 growing at 37°C, about 3% of the total RNA is messenger with a half-life of 2.7 to 3.0 min (214). The stability of mRNA in exponential-phase cultures of *B. amyloliquefaciens* growing at 30°C has been estimated to have a half-life of 4.5 min (30). The relationship between mRNA half-life and growth temperature has not been determined for *B. amyloliquefaciens*, but in *E. coli* the mRNA half-life of 2.9 min in cells growing at 35°C is increased to 4.2 min in cells incubated at 30°C in the same medium (98). Assuming a similar temperature dependence in bacilli, then the results for *B. subtilis* and *B. amyloliquefaciens* are in good agreement.

The stability of mRNA in postexponential-phase cultures of *B. amyloliquefaciens* at 30°C has been determined as a function of rifampin resistant leucine incorporation. A half-life of 6 min was obtained (30). To explain this apparent increased stability of the mRNA in postexponential-phase cells, it was suggested that the mRNA for extracellular enzyme synthesis may have double the half-life of cellular protein mRNA. In exponential-phase cultures of *B. amyloliquefaciens*, exoenzyme mRNA represents a very small fraction of the total mRNA and, thus, the extra stability would have little effect on the average figure of a 4.5-min half-life. However in postexponential-phase cells exoenzyme and cell-protein mRNA are present in equal amounts. The 6-min half-life of the mRNA from these cells could therefore be composed of 50% with a half-life of about 8 min (exoenzyme mRNA) and 50% with a half-life of about 4 min (cell protein mRNA). In support of this hypothesis Brown and Coleman (30) point out that the decay characteristics of phase 1 protease synthesis in amino acid-repressed *B. amyloliquefaciens* follows an exponential curve with a half-life of about 9 min (96). However, this figure is probably inaccurate because it is most unlikely that amino acid repression would produce a complete cessation of transcription of the protease gene and the figure will thus be erroneously high. In fact Elliot's group predicts a short half-life for protease mRNA of only a few minutes from their studies (94, 96). Thus, there is no evidence for an increased stability of exoenzyme mRNA but nevertheless the different half-lives of exponential- and postexponential-phase mRNA (as measured by the potential for protein synthesis after inhibition of transcription) remains to be explained. It is possible that the high concentration of rifampin used in these studies could have a nonspecific effect on the translational abilities of post-exponential-phase *B. amyloliquefaciens* that is absent from exponential-phase cells. Alternatively, there may be some change in the translational apparatus in stationary-phase cells, many examples of which are known to occur in sporulating *B. subtilis* (24).

Brown and Coleman (30) have measured the stability of mRNA in *B. amyloliquefaciens* by using hybridization of DNA to RNA and provided estimates of mRNA half-lives in both exponential- and postexponential-phase cells of 6 min. This figure is slightly higher than that obtained from the leucine incorporation experiments and indicates a constant mRNA stability

in both growing and stationary-phase cells. Thus, the evidence suggests that the mRNA responsible for exoenzyme synthesis has a short half-life of between 4.5 and 6 min, and an exceptionally long-lived mRNA species coding for exoenzymes, as has been suggested in the past, is excluded.

Substrate Induction

In the preceeding discussion of exoenzyme regulation it has been assumed that these enzymes are synthesized constitutively. The situation is, however, more complex, and the available evidence suggests that exoenzyme synthesis may be inducible, partially constitutive, or completely constitutive depending on the bacterial strain and enzyme in question. At least two of the exoenzymes of the genus, levansucrase (LS) and penicillinase, fall into the first category, in that the addition of inducer to the culture increases the differential rate of synthesis of the enzyme from a low basal level to a relatively high, fully induced level.

LS is one of two enzymes involved in sucrose metabolism in *B. subtilis*. It is believed to be at least partially extracellular (244). During exponential growth on glycerol LS activity is very low. The addition of 10^{-4} M sucrose induces synthesis of intracellular sucrase but a higher inducer concentration (10^{-2} M sucrose) is required for LS synthesis, which responds as a typical inducible catabolic enzyme (244).

The regulation of penicillinase synthesis has been studied in considerable detail. Penicillinase synthesis in *B. cereus* is induced by the addition of benzylpenicillin (2 U/ml) to the culture, but the response is not immediate. It was once thought that there was a lag period of about 10 min (112) between the time of addition of inducer and the initial increase in penicillinase activity, but a reexamination of the kinetics of induction has reduced this lag period to 30 s (135). The rate of penicillinase accumulation increases thereafter following a smooth continuous timepath for at least 30 min after the addition of inducer. The penicillinase system is possibly unique in that the inducer does not enter the cell but is bound to the cell wall (5). Thus, the increase in the rate of penicillinase synthesis that occurs more than 5 min after the addition of inducer results from the expression of previously "bound" inducer and not from a greater uptake of inducer. In fact, sufficient penicillinase has normally accumulated in the medium after 5 min to destroy essentially all of the exogenous penicillin (135). Aten and Day (5) have solubilized bound penicillin from the cell walls of *B. cereus* and shown that the release of penicillin is accompanied by decreased penicillinase synthesis. Unfortunately, there was a loss of cellular metabolic integrity during the solubilization procedure, so this observation may not be entirely valid.

The majority of exoenzymes synthesized by the bacilli appear to be at least partially inducible. Catabolic enzymes are normally induced by the enzyme substrate, but exoenzymes are an exception in that they are presumably excreted because the substrate is unable to enter the cell. The substrate cannot, therefore, be directly involved in the induction process although it is conceivable that it could indirectly induce the enzyme via a cell wall-membrane binding site, as in the example of penicillinase. It is now generally accepted however, that a low basal level of constitutive exoenzyme degrades its exogenous substrate and the resultant low-molecular-weight products enter the cell and induce further exoenzyme synthesis.

α-Amylase synthesis is regulated in some bacilli by such a mechanism, and maltotetraose is the most effective inducer of this enzyme in both *B. stearothermophilus* (331) and *B. licheniformis* (269). Maltotriose and the higher maltooligosaccharides (G5, G6, and G7) are less efficient inducers than maltotetraose, and oligosaccharides unrelated to starch, e.g., melibiose and cellobiose, are very poor inducers. These findings explain the observation of the industrial microbiologist. It has long been known that higher yields of α-amylase are obtained from bacteria grown on medium containing complex starch materials than on artificial defined media (36, 236). Not only do these carbon sources fail to exert catabolite repression, but it would seem that they also provide maximal induction of α-amylase. However, some bacilli appear to synthesize α-amylase constitutively. Thus, *B. amyloliquefaciens* (46), *B. subtilis* 168 (287), and *B. licheniformis* (211) have been reported to synthesize α-amylase in the absence of α-1,4-linked oligosaccharides. Unfortunately, the effect of maltooligosaccharides on the differential rate of α-amylase synthesis in these bacilli has not been determined, and it is possible that a relatively high basal level of constitutive enzyme is being secreted that could be increased by induction.

The synthesis of β-amylase by *B. polymyxa* occurs only in the presence of starch or a starch-like product (103). Maltose, the major end product of the exo-attack of amylose by β-amylase yields only 50% of the enzyme activity produced when starch is supplied as the carbon source. It is possible that the β-limit dextrin produced by

the β-amylolysis of amylopectin, or some small maltooligosaccharide derived from the synergistic action of the β-amylase and α-1,6 starch-debranching enzyme (104) may play some part in the inductive process of both these enzymes. Like β-amylase, the cyclodextrin glucosyltransferase (CGT) of *B. macerans* is produced maximally in starch-containing media, and the enzyme accumulates as the concentration of the carbon source declines (177).

In addition to the starch-degrading enzymes, *B. polymyxa* secretes a xylanase (83) and a laminarinase (75) both of which are, at least partially, inducible. Xylanase synthesis is negligible when cultures are grown on a starch-peptone medium but increases dramatically upon the addition of xylan (83), and, similarly, laminarinase synthesis is greatest when laminarin is supplied as the carbon source (75). In their studies of exo-β-*N*-acetylglucosaminidase synthesis by *B. subtilis*, Brewer and Berkeley (28) found that the enzyme is synthesized constitutively during the late exponential phase of growth but that the yield may be increased by the addition of cell wall fragments. Furthermore, the addition of lysozyme to a culture induced exo-β-*N*-acetylglucosaminidase at any time during the growth cycle suggesting that it is the cell wall hydrolysis products that effect the induction (241). In general, the pectinases of the bacilli are inducible enzymes and high yields are obtained when pectin or polygalacturonic acid is provided as carbon source (61, 144).

One bacterium may synthesize both constitutive and inducible exoenzymes with similar functions. Thus, *B. subtilis* K50 secretes an inducible arabinogalactanase (77) and a constitutive mannanase (78), but perhaps the most complex system of inducible exoenzymes among the bacilli is the variety of glucanases and related enzymes elaborated by *B. circulans* WL-12 (167, 168, 262, 263, 310). The β-1,3-glucanase activity in the culture medium of *B. circulans* WL-12 can be resolved into at least six fractions by polyacrylamide gel electrophoresis (167). The relative amounts of all six of these enzymes differ markedly depending on the nature of the carbon source, whether it is *Piricularia oryzae* cell walls, *Saccharomyces cerevisiae* cell walls, or pachyman. In particular, β-1,3-glucanase F1-A is especially lytic toward *P. oryzae* cell walls and is only synthesized in response to *P. oryzae* mycelium as the carbon source. This organism also synthesizes at least four chitinases when grown on crab-shell chitin, and these seem to be controlled by a similar complex induction system (310). The term "concerted induction" of enzymes has been proposed for this phenomenon (310).

In conclusion, although the induction of exoenzymes undoubtedly occurs, the evidence is largely empirical and often ill defined. Only in the examples of α-amylase and exo-β-*N*-acetylglucosaminidase synthesis has it been firmly established that the hydrolysis product of the enzyme's action effects the induction, although in many instances this mechanism has been assumed. The mechanism of induction remains completely unknown, and in no instance has a nonmetabolizable, gratuitous inducer been described. Such a molecule would be an invaluable aid in the elucidation of the induction process.

End Product Repression

The products of exoenzyme action may repress the synthesis of the enzyme in a manner resembling the end product repression of endocellular anabolic enzymes. Extracellular protease synthesis is regulated in this way and is strongly repressed by the presence of amino acids or peptides in the environemnt (71). Individual amino acids do not repress with equal efficiency nor do the same amino acids cause effective repression in the various bacilli. Late exponential-phase cells of *B. amyloliquefaciens* continue to synthesize protease when suspended in medium containing low concentrations of casein hydrolysate (0.25 mg/ml), but severe repression of the enzyme(s) is caused by higher (0.5 mg/ml) concentrations (204). Of 16 amino acids examined, proline, isoleucine, glutamate, and aspartate were found to produce the most severe repression. In *B. megaterium*, neutral protease synthesis is inhibited by threonine and isoleucine (40). Similarly in *B. licheniformis* (14) and *B. cereus* (185) protease synthesis is repressed by high extracellular amino acid concentrations, and RNase synthesis in *B. amyloliquefaciens* is repressed by inorganic phosphate (205, 303). In all these examples the mechanism of the repression remains obscure.

GENETIC ANALYSIS

Any attempt to collate the relatively few genetic studies of exoenzyme synthesis in *B. subtilis* is immediately beset with difficulties. The main problem that is less troublesome in studies of intracellular enzyme systems is pleiotropy, and the full phenotypes of many mutants deranged in their control of sporulation and/or exoenzyme synthesis remain unknown. Many of these mutations have not been linked to other genetic markers, and their map positions

have not been determined. It will be important to map the locations of these markers in future so that a genetic comparison of similar phenotypes can be made. The genetic loci that have been reported to be involved in exoenzyme synthesis have been listed in Table 2, and Fig. 1 shows the approximate map locations of the linked markers.

α-Amylase

The α-amylase (*amy*) cistron of *B. subtilis* was first analyzed in detail by Yuki and Ueda (353), although the α-amylase structural gene had been recognized as a transformable element some 6 years previously (100). Transformational analysis of the cistron was facilitated by the isolation of two linked markers, *phe-96* and *aro-116*. By using these markers Yuki and Ueda (353) placed 16 Amy⁻ mutants in a single locus, *amyE*, on the *B. subtilis* 168 chromosome. *amyE* is the structural gene for α-amylase (353). Similarly, Yamaguchi et al. (342) isolated 28 Amy⁻ mutants in which α-amylase was undetectable in 11 and reduced in the remaining 17. Every *amy* marker was found to be cotransformable with *aro-116* at a frequency of 30 to 40%. Three-point crosses were used to place *aro-116* to the right of *amyE*, and a tentative genetic map was proposed. The enzymological properties of four thermosensitive α-amylases from strains possessing point mutation in *amyE* have been studied in detail and compared with the parental α-amylase (345). More recently, the *amyE* region has been placed on the genetic map of *B. subtilis* as revised by Lepesant-Kejzlarová et al. (184). *aro-116* has been shown to lie in the *aroI* cistron (352), which has been located on the early replicating portion of the chromosome between *lin* and *narB* (111; Fig. 1). Three-point transformation crosses have been used to confirm the location of *amyE* and provide a map order, *lin amyE aroI narB* (352).

The yield of saccharifying α-amylase from *B. subtilis* Marburg, *B. subtilis* NAT, and *B. subtilis* SAC of 10, 50, and 150 U per mg of cells, respectively, reflects the relative rates of synthesis of this enzyme in these strains (348). The α-amylases from *B. subtilis* and *B. subtilis* NAT can be differentiated according to their electrophoretic mobilities. A character that determines the high level of α-amylase synthesis in *B. subtilis* NAT can be transferred to *B. subtilis* by transformation. About 10% of the transformants with the Amy^h phenotype produce the recipient-type enzyme, Marburg (M) α-amylase, and the remaining 90% produce the donor type, NAT (N) α-amylase (340). Further

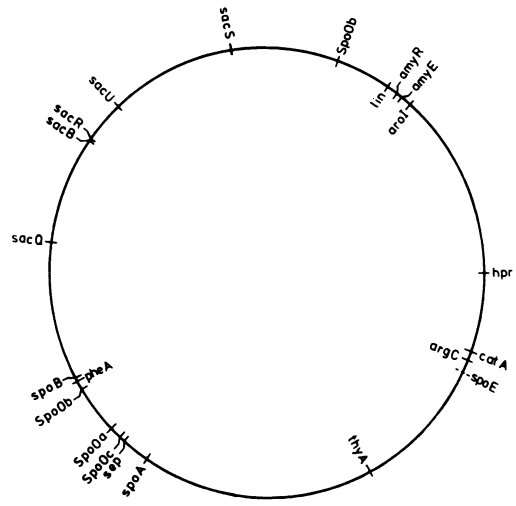

FIG. 1. *Linkage map of B. subtilis based on the revised location of markers reported by Lepesant-Kejzlarová et al. (184). Loci affecting the synthesis of extracellular enzymes are placed on the outside of the circle; reference markers on the inside.*

genetic analysis has shown that (i) the structural genes *amyEm* and *amyEn*, for types M and N α-amylases, respectively, are allelic to each other and (ii) a gene, closely linked to the structural gene, regulates the rate of α-amylase synthesis. This gene has been designated *amyR* where *amyR1* of *B. subtilis* and *amyR2* of *B. subtilis* NAT direct a low and high rate of α-amylase synthesis, respectively. It follows that, theoretically, four α-amylase-producing strains with different phenotypes could be constructed by transformation. The genotypes would be: *amyEm amyR1; amyEm amyR2; amyEn amyR1; amyEn amyR2*. Yamaguchi et al. (341) have prepared four such strains and studied them in detail. It was found that the α-amylase of isogenic strains carrying *amyR1* or *amyR2* were serologically and biochemically identical regardless of the types of enzyme. Therefore, the *amyR* locus could not be part of the *amyE* gene possibly affecting the specific activity of the enzyme but must, in fact, be a control element dictating the rate of synthesis of the enzyme. Furthermore, *amyR* was shown to have no effect on the production of either extracellular protease or RNase but was specific for α-amylase (341). The relative location of *amyR* to *amyE* has been determined by genetic crosses between *amyE* mutants with *amyR1* or *amyR2* strains. The results (342) showed that *amyR* maps to the left of *amyE* in the order *lin amyR amyE aroI narB*, thus confirming the order originally pro-

posed by Yuki (351). The distance between *amyR* and *amyE* has not been accurately determined but it is far smaller than the average gene, and it seems probable that *amyR* adjoins to a terminal region of *amyE*. Yamaguchi et al. (341) suggest that *amyR* may be the promoter region from which transcription of *amyE* proceeds, but further genetic studies are necessary before any specific function can be assinged to the *amyR* locus.

DNA from *B. subtilis* Marburg and *B. subtilis* SAC is 85% homologous, and DNA from the latter strain transforms *B. subtilis* 168 with high efficiency. Yoneda et al. (348) exploited this observation by transforming *B. subtilis* NA20-22, a strain of *B. subtilis* 168 carrying the α-amylase structural gene from *B. subtilis* NAT (*amyEn amyR1*), with DNA from *B. subtilis* SAC. Transformants producing high levels of α-amylase were selected. Of 27 such transformants, 25 produced the donor-type α-amylase (type S) and two produced the recipient (type N) α-amylase. All 27 transformants produced 30 to 50 U of α-amylase per mg of cells, characteristic of strains carrying *amyR2*. The *amyR* allele of *B. subtilis* SAC has been designated *amyR3* (348). It does not differ phenotypically from *amyR2*, but because these alleles originated from natural sources in different strains it seems reasonable to assume that they have different nucleotide sequences. Further genetic studies have shown that (i) *amyR3* is closely linked to *amyEs* and that both genes are linked to *aroI116* and (ii) the gene order around the α-amylase cistron in *B. subtilis* SAC, *B. subtilis* NAT, and *B. subtilis* Marburg is very similar (348). The genetic determinant(s) conferring the "extra high" production of α-amylase by *B. subtilis* SAC does not therefore lie in the *amyR3* locus but, as described below, may be the result of a gene that affects the rate of synthesis of both α-amylase and protease.

A second gene affecting the quantity of α-amylase produced by *B. subtilis* has recently been described by Sasaki et al. (276). Of seven mutants of *B. subtilis* NA64 (*amyR2*) that were resistant to the antiviral antibiotic tunicamycin (TM), two produced two- to fivefold increased levels of α-amylase. The mutation is specific for α-amylase and has no effect on RNase or protease synthesis. Transformation analysis revealed that the Tm^r Amy^h phenotype was the result of a single mutation in the tentatively named *tmr* gene. This gene did not cotransform with *amyR*. TM selectively inhibits cell wall synthesis in *B. subtilis*, and, thus, the hyperproduction of α-amylase may be the result of an alteration in the structure of the cell surface. The reason for the selective effect on α-amylase remains unknown.

Proteases

B. subtilis secretes three proteases during the early stationary phase of growth of which at least one, the alkaline serine protease, appears to be intimately associated with the process of sporulation. Although this complicates genetic analysis of these enzymes, some advances are being made by employing interstrain transformation as in the genetic analysis of α-amylase synthesis in this organism. *B. subtilis* NAT produces 15 to 20 times more extracellular proteolytic activity than *B. subtilis* Marburg, the difference being due to the amounts of neutral protease (321). The ratio of neutral to alkaline protease activity in the culture medium of *B. subtilis* Marburg is 1:1 and in *B. subtilis* NAT is 1:13. The enzymological and serological properties of the serine and neutral proteases of these two strains are quite similar and identical, respectively.

Transformation of *B. subtilis* 168 with DNA from *B. subtilis* NAT (321) resulted in 81 transformants screened for the hyperproduction of neutral protease (Npr^h). These transformants produced almost the same level of total extracellular protease as the donor, *B. subtilis* NAT, and could be divided into three groups: (i) the major class, 69 transformants with the phenotype Npr^h Sep^+ Amy^+; (ii) one transformant with the phenotype Npr^h Sep^h Amy^+; and (iii) three transformants with the phenotype Npr^h Sep^+ Amy^h. The authors suggest that neutral protease may be regulated by a mechanism similar to that controlling α-amylase synthesis in *B. subtilis* (321). Thus, neutral protease is coded for by a structural gene, *nprE*, the expression of which is dictated by a regulator gene, *nprR*. *nprR2* of *B. subtilis* NAT directs a high rate of neutral protease synthesis and *nprR1* of *B. subtilis* Marburg directs a relatively low rate.

The genetics of serine protease synthesis is complicated by the close association of this enzyme with sporulation. The structural gene for serine protease has been identified by the mutation *ts-5* isolated by Leighton et al. (179, 180). *B. subtilis* WB 746 *ts-5* possesses wild-type properties at 30°C, but at 47°C it is asporogenous and produces a defective serine protease. *ts-5* has been mapped between *aroD* and *lys* by PBS1 transduction (179), but doubt remains as to whether the ts Spo0 and ts Sep^- phenotypes are the result of a single mutation because Millet et al. (221) have succeeded in separating

these characters by transformation (see section "Physiological Functions"). Thus, despite the extensive evidence of Leighton et al. (179, 180), the involvement of the serine protease in sporulation is unconfirmed. Specific mutations increasing the yield of serine protease have not been found, although many pleiotropic mutations increasing the yield of serine protease and other exoenzymes have been reported (see below).

LS

LS differs from both α-amylase and the proteases in that it is a fully inducible enzyme synthesized in response to sucrose in the environment. A number of mutants representing three different Lvs phenotypes have been isolated: (i) Lvs$^-$ strains produce less enzyme than their parent *B. subtilis* 168; (ii) Lvs^c strains produce the normal amount of enzyme constitutively; and (iii) Lvs^h strains produce increased (100-fold) amounts of LS inducibly. Lvs$^-$ mutants have been mapped in three loci by PBS1-mediated transduction (182): *sacB*, *sacS*, and *sacU* (Fig. 1). To identify the structural gene for LS, Lepesant et al. (183) characterized the enzyme from two mutants affected in either temperature sensitivity or catalytic properties. Both were shown to be point mutations of the *sacB* gene, strongly suggesting that this is the structural gene for LS and that the Lvs$^-$ phenotype arising from lesions in *sacU* and *sacS* must be control dysfunctions. Seven mutants constitutive for LS have been mapped by PBS1 transduction and found to lie in a cluster between *cysB3* and *hisA1*, the *sacR* locus (182). Ten remaining Lvs^c mutants did not cotransduce with *hisA1* and were placed in *sacS*. *sacS*^{c} mutants are constitutive for both LS and intracellular sucrase. A total of 23 Lvs^h mutants were mapped in three distinct groups by PBS1-mediated transduction (182): *sacS*^{h}, *sacQ*^{h}, and *sacU*^{h}. The *sacQ*^{h} and *sacU*^{h} mutations are pleiotropic and are discussed more fully below. *sacS*^{h} mutants hyperproduce only LS. There has been little functional analysis of the LS system; therefore, it is difficult to assign roles to the individual loci, but *sacR* may be analogous to the *amyR* and *nprR* cistrons.

Pleiotropic Mutations

By far the majority of mutations affecting exoenzyme synthesis are pleiotropic, either for more than one exoenzyme or possibly involving motility, control of sporulation, and several other seemingly unrelated events (Table 2). While screening for Amy^h mutants of *B. subtilis*, Yoneda et al. (347) observed that of 15 strains with the Amy^h phenotype, six produced increased levels of both α-amylase and protease and the remaining nine strains produced increased levels of α-amylase only.

These six pleiotropic mutants secreted two to three times more α-amylase and four to sixteen times more protease than the parent, *B. subtilis* Marburg. The α-amylase synthesized by the mutants was biochemically and serologically identical to the parental enzyme, and the increased proteolytic activity was due to overproduction of both the serine and neutral enzymes, although the increase in the former was greater. Cell growth and the synthesis of RNase and several intracellular enzymes were unaffected, although the level of extracellular alkaline phosphatase was considerably reduced. The mutation responsible for this phenotype was shown to lie in a single gene by transformation experiments in which transfer of Amy^h was invariably accompanied by *prt*^{h} and vice versa. The mutation did not cotransform with *aroI116* and was therefore not a lesion in *amyR* (346). The gene has been designated *pap* (production of α-amylase and protease [346, 347]).

When the *pap* mutation and *amyR2* coexist in the same strain the two genes are expressed synergistically and the organism produces as much as 150 U of α-amylase per mg of cells together with about 60 U of protease per ml. These figures are typical of the enzyme quantities synthesized by *B. subtilis* SAC, and this observation prompted Yoneda et al. (348) to examine the significance of *pap* in the synthesis of extracellular enzymes in this organism. These authors transformed *B. subtilis* NA20 (*amyR2*) with a saturating concentration of DNA from a streptomycin-resistant strain of *B. subtilis* SAC and selected for Str^r transformants. Among these Str^r transformants, strains hyperproducing protease were identified on casein plates and the synthesis of α-amylase examined for 19 such Prt^h transformants. Only three α-amylase extra-high producers were found, indicating that the characters for extra-high α-amylase and high protease production in *B. subtilis* SAC segregate as separate markers and that *pap* is not involved. The mechanisms involved in the regulation of α-amylase and protease synthesis in *B. subtilis* SAC have yet to be elucidated.

Nevertheless the *pap* gene is interesting in itself as a pleiotropic phenomenon. Ayusawa et al. (8) have extensively characterized the phenotype of the *pap* mutant *B. subtilis* YN9. This organism grows and sporulates normally but possesses a markedly reduced autolytic activ-

ity (Lys$^-$). This latter trait is the result of a decreased autolytic enzyme content rather than a reduced susceptibility of the cell wall to autolysis. In addition, strain YN9 is nontransformable due to an inability to become competent and lacks flagella (Mot$^-$). A pool of flagellin can be detected serologically in the cytoplasm, suggesting that the lack of flagella is the result of an inability to polymerize the flagellin molecules or some similar deficiency. The protein spectra of the cytoplasmic membranes of wild-type and *pap* strains have been compared by sodium dodecyl sulfate-discontinuous gel electrophoresis. A protein band, present in the wild-type membrane preparation, is absent from the *pap* membrane. However, the mobility of this band is similar to that of purified flagellin, and it is conceivable that the band in the wild-type membrane preparation was due to contamination by flagellin. Thus, it remains unclear whether the *pap* mutation affects the cytoplasmic membrane or not, although chemical data suggest that there is no essential difference in the composition of *pap*$^+$ and *pap* membranes.

The *pap* mutation is by no means unique. Sekiguchi et al. (288) sequentially mutated *B. subtilis* 168 with ultraviolet light and isolated two Amy^h mutants; strain 128 produced α-amylase and neutral and alkaline proteases in the ratios 2×, 0.25×, and 0.25× and strain 196 produced the above 4×, 5×, and 5× the parent, respectively. In addition to the Amy^h Prt^h phenotype *B. subtilis* 196 lacked flagella. A single pleiotropic mutation named *amyB* was deemed responsible from transformation analysis. Mutation in a second locus, *amyC* was believed to result in the production of strain 128 from the wild type, but the relationships between *pap*, *amyB*, and *amyC* are unknown. (*amyB*, *pap*, and *sacU*^{h} have now been shown to be identical [300a].)

A third group of mutants that phenotypically resemble both *pap* and *amyB* mutants are strains bearing lesions of the *sacU* locus (170). *sacU* strains are characterized by a deficiency in LS and protease synthesis whereas *sacU*^{h} mutants hyperproduce both serine and neutral proteases and LS. In addition, they may or may not lack flagella and, unlike the parent, sporulate in rich media (control sporulation defective; Csp$^-$). A second mutant class, the *sacQ*^{h} mutants, are less pleiotropic and hyperproduce only LS and protease. Both *sacU*^{h} and *sacQ*^{h} are single mutations (170).

Mutants of Prt^h Csp$^-$ phenotype are not uncommon. Ito and Spizizen (138) have described a mutant of *B. subtilis* 168 that sporulates in the presence of glucose and simultaneously hyperproduces proteases. This mutant has a lesion in the *catA* locus and is not only genetically different from *sacU*^{h} *sacQ*^{h} mutants but also synthesizes wild-type levels of LS (170). Similarly, protease hyperproducers of *B. cereus* have been isolated that, unlike the wild type, sporulate in rich media (185). However, mutants of the Prt^h phenotype need not necessarily be altered in sporulation control. The *hpr* mutants characterized by Higerd et al. (120) synthesize increased amounts of both neutral and serine protease but normal levels of α-amylase. Similar mutants have been described by Balassa et al. (10). The *hpr* locus has been placed between *argC4* and *hisA1* by PBS1-mediated transduction (Fig. 1).

Penicillinase

Genetic analysis in *B. licheniformis* was given a considerable impetus in the mid-1960s by the development of a high-frequency transformation system in strain ATCC 9554A. Fortunately, DNA from strain 749, which is used for much of the penicillinase biochemistry, efficiently transforms strain ATCC 9554A. By using such a system, Sherratt and Collins (292) extended the genetic analysis of penicillinase synthesis instigated by Dubnau and Pollock (73). After tests with a variety of markers, the *pen* gene was found to be over 2% cotransformed with *ilvD1*, thus providing sufficient linkage for preliminary genetic analysis (292). Two- and three-factor transformation crosses subsequently revealed a gene order *ilvD ilvA pen*. Eight penicillinase structural gene (*penP*) mutants were mapped either close to or between *P22* and *P72*, both of which synthesized no detectable penicillinase activity but produced serologically cross-reacting material. Regulatory mutants have been mapped in at least two loci. One regulatory gene, *penI* is 90% linked to *penP* and is the region from which mutants with magnoconstitutive phenotypes are derived. Two other regulatory mutants, *R27* and *E14*, were partially inducible and 50% linked to *penI*. A third type of regulatory mutation, *R32*, was located on the opposite side of *penP* to *penI* and resulted in defective inducibility and decreased expression of *penP*. A frameshift mutation, *penP102* has been shown to map at the distal end of *penP* adjacent to *penI* (158). This mutation is polar on *penI*, suggesting that the *penP* and *penI* genes are in an operon that is read in the direction $P \rightarrow I$ (158). These findings have been composed into a model for the regulation of penicillinase synthesis in *B. licheniformis* (292). It is proposed that the control of the "operon" is exerted through a repressor protein coded in *penI* and

an effector molecule (identified by *R14* and *R21*), which together bind at *penO* (identified by *R32*). It is envisaged that repression by the *I* gene product is either aided or antagonized by the effector molecule, whose concentration is affected by events such as penicillin attachment to the penicillin binding site.

MECHANISM OF SECRETION

Despite the relative complexity of the eucaryotic organism, it is with these cells that the major advances in our understanding of the transport of proteins across membranes have been achieved. Consequently, I shall discuss the proposed mechanisms of exoenzyme secretion in the procaryote in the context of the eucaryotic system.

Eucaryotic cells contain two distinct populations of ribosomes; those free in the cytosol and those bound to membranes. The membrane-associated ribosomes are predominant in secretory cells, including pancreatic acinar cells, plasma cells, and hepatocytes (90). In the mid-1950s Palade suggested that these membrane-bound ribosomes are the site of synthesis of proteins that are to be secreted. More recently it has been demonstrated that the ribosomes bind to the membrane through the larger of the two ribosomal subunits and that the polypeptide chain is vectorially discharged from the membrane-bound ribosome through the membrane into the intraluminal compartment of the endoplasmic reticulum (253, 266). Although this appears the most reasonable and efficient mechanism by which a cell, either eucaryotic or procaryotic, can externalize a protein, it must be emphasized that conclusive evidence for the synthesis of bacterial exoenzymes on membrane-bound ribosomes is lacking. Nevertheless, it is difficult to construct a plausible scheme for exoenzyme secretion in the absence of membrane-bound ribosomes.

As described earlier, a number of observations suggest that procaryotic exoenzymes do not occur, at least in their native configuration, in the cytoplasm, thus supporting the proposal that they are secreted as they are synthesized. Furthermore, in many bacteria a considerable but variable fraction of the ribosomal population has been reported to be associated with the cytoplasmic membrane. In exponential-phase *B. licheniformis* cells 96% of the total ribosomal material is membrane bound, and no polysomes are present in the cytosol (69). In *B. megaterium* (284) and *B. amyloliquefaciens* (47) 35 to 50% of the ribosomal material is associated with the membrane. Thus, a population of potential exoenzyme-synthesizing ribosomes does exist in these bacteria. Coleman (48) studied the distribution of α-amylase-forming ability between the soluble and membrane-bound ribosomal fractions of *B. amyloliquefaciens*. Both types of ribosome incorporated labeled amino acids equally efficiently. However, the synthesis of α-amylase was fivefold greater in the membrane-bound fraction than in the soluble fraction, suggesting that the membrane-bound polysomes were preferentially synthesizing exoenzymes. Unfortunately, no conclusive differences in activity were observed.

If we therefore assume that procaryote-secreted proteins are synthesized on membrane-bound ribosomes, as are their eucaryotic counterparts, a major questions is, how does the cell decide which proteins must be transported across the membrane, or alternatively, how do individual mRNA molecules decide between membrane-bound and soluble ribosomes? In answer to these questions Blobel and Sabatini formulated the "signal" hypothesis for eucaryotic cells (19, 20), and evidence is now emerging to suggest that a similar system may also operate in procaryotes. The signal hypothesis proposes that mRNA's for secretory proteins possess a unique sequence of codons located immediately to the right of the initation codon. These codons, the signal codons, are not present in the mRNA's coding for cytoplasmic proteins. Translation of the signal codons results in a unique amino acid sequence on the amino terminal of the polypeptide chain, the signal sequence. When the nascent polypeptide chain bearing the signal sequence emerges from the large ribosomal subunit, it is recognized by two or more membrane receptor proteins, causing their loose association to form a tunnel in the membrane. At the same time, the sequence may dissociate one or more proteins on the large ribosomal subunit revealing an attachment area. The ribosome would then bind to the membrane receptor proteins stabilizing the tunnel and providing a confluent passage from the ribosome through the membrane. After completion of the nascent polypeptide chain, ribosomal detatchment from the membrane would eliminate the cross-linking effect of the ribosome on the membrane receptor proteins, and these would be able to diffuse freely in the plane of the membrane. After the nascent polypeptide has traversed the membrane, it is proposed that an endopeptidase specifically removes the signal sequence, thus allowing the enzyme to assume its native conformation (Fig. 2).

One implication of the signal hypothesis is that exoproteins are synthesized as oversize

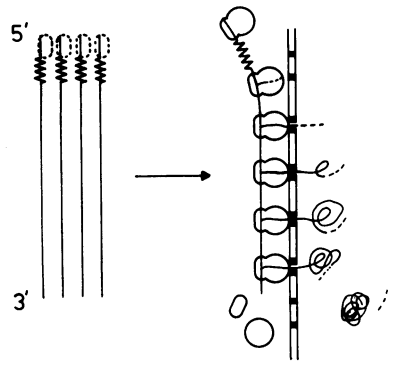

FIG. 2. *Hypothetical model for the transfer of proteins across membranes based on the models devised by Both et al. (23) for procaryotic cells and by Blobel and Dobberstein (19) for eucaryotic cells. The signal codons, after the initiator codon, are indicated by a zig-zag region in the mRNA. The signal sequence of the nascent chain is indicated by a dashed region. It is proposed that exoenzyme mRNA migrates from a nuclear transcription site towards the membrane. In late stationary-phase cells a pool of migrating mRNA accumulates (see text). The messenger may be associated with a migratory protein, possibly the 30S ribosomal subunit (23). As the signal sequence emerges from the 50S ribosomal subunit, it is recognized by membrane proteins. Recognition results in a loose association of these proteins, which is subsequently stabilized by interaction of sites on the large ribosomal subunit with sites on the membrane proteins. After vectorial discharge of the nascent chain through this membrane "tunnel" the enzyme assumes its native configuration. Endoproteolytic removal of the signal sequence results in the formation of stable hydrophilic extracellular enzyme.*

molecules because of the presence of the signal sequence. Experimental results confirm this prediction. The product of the in vitro translation of mRNA for immunoglobulin light chain is larger by about 20 amino acids than the authentic light chain (19). Similarly, it has been shown that mRNA's from calf thymus glands are translated by a wheat-germ extract into preparathyroid hormone that migrates more slowly in sodium dodecyl sulfate-polyacrylamide gel electrophoresis than parathyroid or proparathyroid hormone. Here also, the translation product is larger than the normal protein by about 20 to 25 amino acid residues (159). A second implication is that significant homology, if not complete identity may be expected in the signal sequences of different exportable proteins. Devillers-Thiery et al. (68) determined the amino acid sequence of up to 24 amino acid residues for several putative precursors for dog pancreas secretory proteins. Extensive homology and a high proportion of hydrophobic residues were revealed in the 16 amino-terminal residues.

Returning to the bacilli, Dancer and Lampen (57) studied the in vitro synthesis of penicillinase by cell-free extracts of *B. licheniformis* 749/C, and showed that polyribosomes from this source exclusively synthesized the membrane-bound type of penicillinase. There was a negligible yield of the hydrophilic enzyme. The membrane-bound penicillinase of *B. licheniformis* is larger than the hydrophilic extracellular enzyme by a sequence of 25 amino acid residues terminating with a phospholipid group (344). This lipopeptide is attached to the amino-terminal end of the polypeptide and, although the peptide is polar, the phospholipid group confers a hydrophobic character to the enzyme. It is very tempting to speculate that this lipopeptide is a procaryotic signal sequence. A membrane-associated from of α-amylase has recently been purified from the cytoplasmic membrane of *B. amyloliquefaciens* (260). It is larger than the extracellular enzyme and differs in both electrophoretic mobility and sedimentation velocity. This also may be an extracellular molecule bearing an intact signal sequence; the examination of more exoenzyme precursors and, in particular, the amino-terminal sequences may prove these speculations.

A critical feature of the model is that if the enzyme traverses the membrane in an incompletely folded form to adopt its native configuration exterior to the membrane, it is likely that directly upon release it will be protease sensitive, later becoming protease resistant. Such evidence for a partially folded intermediate of penicillinase in *B. licheniformis* has been reported by Bettinger and Lampen (16). These authors produced protoplasts of *B. licheniformis* that had been stripped of approximately 50% of their membrane-bound penicillinase by treatment at high pH or with trypsin. The protoplasts produced active penicillinase in its normal, protease-resistant configuration when suspended in hypertonic growth medium. The addition of trypsin or chymotrypsin (25 μg/ml) to the system prevented penicillinase accumulating, an effect that was rapidly reversed by the addition of soybean-tryspin inhibitor. These findings suggest that the protease degrades the nascent enzyme at some point during the secretion process before it can assume its normal protease-resistant conformation. Analysis of the supernatant fluid from chymotrypsin-treated, penicillinase-secreting protoplasts revealed peptides derived from the degraded penicillinase substantiating the interpretation. Similar evidence for incompletely folded secretory forms of α-amylase and pro-

tease from *B. amyloliquefaciens* has been obtained (271). Excretion of active α-amylase and protease by protoplasts of *B. amyloliquefaciens* is totally inhibited by the presence of proteolytic enzymes including trypsin, chymotrypsin, and the purified extracellular protease from *B. amyloliquefaciens* itself. As in the case of penicillinase the exoenzymes in solution are stable to proteolysis, and only the enzyme emerging from the protoplast is restricted. Removal of the protease allows the excretion of active exoenzyme to continue.

The observations of Sanders and May (271) highlight a significant problem in the process of secretion, namely that the incompletely folded form of the secreted enzyme is susceptible to proteolysis by the organism's own extracellular protease(s) which is presumably undergoing a similar conformational change on the exterior of the membrane. The emerging enzymes must therefore fold into their native configuration in a protected environment of some kind. In this connection Bettinger and Lampen (16) found that tryspin only prevented the secretion of penicillinase when the level of bound penicillinase was reduced by 50% or more. This was achieved by either prestripping the protoplasts in alkaline medium or suspending intact protopalsts in sufficient trypsin that the level of bound enzyme was reduced. The authors surmised that a combination of bound enzyme and other molecules in the outer surface of the protoplasmic membrane was required to protect the initial form of penicillinase during its adoption of a resistant conformation. In addition, it is notable that the bound membrane form of penicillinase is always maintained at a constant level by preferentially retaining the nascent penicillinase as bound enzyme at the expense of exopenicillinase formation (274). However, stripped protoplasts can only reincorporate that amount of enzyme that has been removed, indicating that the membrane can accommodate only a fixed amount of penicillinase and that the organization of penicillinase binding sites on the membrane is critical. Although, as previously mentioned, the in vitro synthesis of penicillinase results in only the membrane-bound enzyme and negligible amounts of the exoenzyme, the location of penicillinase on the membrane is not a prerequisite to excretion. Crane et al. (55) incubated ^{14}C-labeled *B. licheniformis* cells in unlabeled medium at pH 6.5 (chosen to favor retention of newly formed enzyme). Some 15 to 20% of the labeled, membrane-bound penicillinase was subsequently released, but, by far, the major portion of the exoenzyme recovered from the culture medium was newly synthesized, suggesting that bound molecules are not expelled by the de novo hydrophobic-type penicillinase. Thus, it seems that the phospholipid form of penicillinase is a transient but essential intermediate that may be either retained as a membrane-bound molecule if a binding site is free or proteolytically processed as an exoenzyme in the absence of an available binding site. Similarly a constant level of membrane-bound α-amylase can be detected in extracts of *B. subtilis* Marburg, *B. subtilis* NAT, and *B. subtilis* SAC, irrespective of the final extracellular concentrations of enzyme, suggesting a uniform distribution of α-amylase binding sites (229).

The translation of exoenzyme mRNA by membrane-associated ribosomes may clarify the problem of the vectorial transport of proteins, but it introduces its own difficulty, that of mRNA migration. In such a scheme it is necessary for the exoenzyme mRNA, transcribed from a single genetic locus, to be translocated to a multiplicity of translation sites situated on the cytoplasmic membrane. It is known that early stationary-phase cultures of *B. amyloliquefaciens* (23) and *B. subtilis* (289) continue to synthesize exoenzymes for prolonged periods after the cessation of transcription. On this basis it was suggested that these cells contain a pool of mRNA, the result of an imbalance in the rates of transcription and mRNA degradation. Thus, a slightly excessive rate of transcription would lead to the accumulation of an mRNA pool. But it has been emphasized that this pool is not an obligatory intermediate in the process of exoenzyme mRNA translocation because, following their depletion, "uncoupled" transcription and translation are resumed. Moreover, it is doubtful that translation is the rate-limiting step in exoenzyme synthesis. The transfer of a single genetic element, the *amyR2* gene of *B. subtilis* NAT to *B. subtilis* Marburg confers the Amy^h phenotype of *B. subtilis* NAT to the Marburg strain. *amyR* is believed to be a promoter region, controlling the rate of transcription of the α-amylase operon (341). Thus, by increasing the rate of transcription, a greater yield of enzyme is obtained, suggesting that in *B. subtilis* Marburg the rate of α-amylase synthesis is limited, not by the availability of translation sites, but by the rate of transcription. Similar arguments can be applied to neutral protease synthesis in *B. subtilis* and the *nprR* gene, which is believed to control the rate of transcription of this operon (321).

Having traversed the membrane, the cell wall poses the final barrier to the complete

externalization of the molecule. Bull (35), in his review of macromolecule secretion, emphasizes the importance of this structure and suggests that it is no longer reasonable to argue that the permeability barrier imposed by the microbial cell wall can be discounted. Washed-cell suspensions of *B. amyloliquefaciens* continue to secrete α-amylase and protease for about 15 min in the complete absence of protein synthesis (97). Gould et al. suggest that this represents a pool of exoenzyme that has accumulated beneath the cell wall, possibly due to restricted diffusion through the wall. Cells in which this pool has been depleted by chloramphenicol treatment restore the pool before subsequent exoenzyme secretion, and pulse-labeling experiments showed that newly synthesized exoenzyme molecules were either immediately released into the environment or equilibrated with the preformed enzyme before excretion (97). The recent demonstration of membrane-bound α-amylase in *B. amyloliquefaciens* (229) permits a reappraisal of these findings. Rather than restricted diffusion through the cell wall the secretion of α-amylase by chloramphenicol-treated cells may represent the release of membrane-bound molecules. This interpretation is consistent with the process of penicillinase secretion in *B. licheniformis* in which vesicular penicillinase is released from chloramphenicol-treated cells and newly synthesized enzyme does not necessarily equilibrate with the preformed enzyme before secretion (272). The gram-positive cell wall therefore remains largely an unknown quantity in respect of the restriction of exoenzyme diffusion.

EXOENZYME SYNTHESIS IN PROCARYOTES

Several aspects of exoenzyme synthesis in bacilli are common to the bacteria in general and suggest a uniform mechanism for exoprotein synthesis and transport in procaryotes. Exoenzymes may be constitutive, partially inducible or fully inducible; there are no common features among either the enzymes or the bacterial genera from which to predict the nature of the regulatory system. Constitutive exoenzymes include the proteases of *Pseudomonas maltophilia* (21) and an *Arthrobacter* sp. (124), the various exoproteins of *Staphylococcus aureus* (141), the α-1,3-glucanase of a *Flavobacterium* sp. (74), and the polygalacturonate lyases of *Aeromonas liquefaciens* (129) and *Erwinia* spp. (223). Inducible exoenzymes include the proteases of *Aeromonas proteolytica* (191) and a *Micrococcus* sp. (206), the agarase of *Cytophaga flevensis* (212), the dextranase of *Cytophaga johnsonii* (140), the β-1,3-glucanase (188) and chitosanase (250) of *Streptomyces* spp., the cellulases of a *Clostridium* sp. (178) and the polygalacturanate lyases of *Erwinia* (358), *Pseudomonas* (357), and *Xanthomonas* spp. (300). There is, however, one common regulatory feature among all these bacteria and enzymes—catabolite repression. The rate of synthesis of exoenzymes appears to be regulated by catabolite repression of either a constitutive or fully induced gene. But although this crystallizes the problem it does little to explain the mechanism behind the regulation. A low intracellular cyclic AMP concentration is probably responsible for the repression of catabolite-sensitive genes in *E. coli* and the addition of exogenous cyclic AMP reverses the glucose repression of many intracellular enzymes and of penicillin acylase, a surface-bound enzyme in this bacterium (89). In the closely related *Serratia marcescens*, mutants that require cyclic AMP for the utilization of various carbohydrates (*cya* mutants) secrete only 10% of the parental extracellular lipase activity. Supplementing the medium with 1.5 mM cyclic AMP restores the ability to produce high levels of extracellular lipase (336). But the addition of exogenous cyclic AMP fails to relieve catabolite repression of extracellular enzyme synthesis in *Vibrio parahaemolyticus* (131), *Psuedomonas maltophilia* (21), *Pseudomonas lemoignei* (302) and *Staphylococcus aureus* (349). In fact, cyclic AMP antagonizes the synthesis of extracellular β-1,3-glucanase in a *Streptomyces* sp. (189). Some of these results may be due to the low cyclic AMP concentrations employed, Smyth and Clarke (298) have recently shown that 30 mM cyclic AMP is necessary to overcome catabolite repression of intracellular amidase synthesis in *Psuedomonas aeruginosa*, but more likely it reflects varying mechanisms governing catabolite repression in procaryotes.

There is a large amount of evidence to suggest that the synthesis of exoenzymes on membrane-bound ribosomes is a general and perhaps key aspect of exoenzyme secretion in procaryotes. Cancedda and Schlesinger (37) have provided evidence that the periplasmic alkaline phosphatase of *E. coli* may be synthesized on polysomes associated with the membrane, and several investigators have observed that exoenzyme synthesis is considerably more sensitive to antibiotic inhibition than general protein synthesis (21, 302). These data have been interpreted as support for a membrane site for extracellular protein synthesis, the membrane-bound ribosomes being more accessible to the antibiotic, but it is by no means conclusive.

Late exponential-phase cultures of *P. lemoignei* (302) and *P. maltophilia* (21) secrete extracellular enzymes in the absence of transcription. This chloramphenicol-sensitive secretion of protein continues for approximately 30 min in the presence of rifamycins, whereas the incorporation of labeled uracil into trichloroacetic acid-precipitable material is immediately inhibited. The stability of the exoenzyme mRNA has not been determined in these studies. Consequently, it is impossible to differentiate between an inherently long-lived mRNA or multiple gene copies of a short-lived mRNA, but in the light of the studies with *B. amyloliquefaciens* and *B. subtilis*, a pool of short-lived mRNA is probably the most accurate interpretation.

Pleiotropic mutations are a common phenomenon in exoenzyme-secreting bacteria and their isolation and characterization may offer a means of elucidating the mechanism of exoenzyme regulation and secretion. Deficiencies in autolysin content have been indirectly implicated in mutants of *Staphylococcus aureus* possessing pleiotropic exoenzyme phenotypes (349), and pleiotropic mutations in *S. marcescens*, which increase the synthesis of certain extracellular proteins and the rate of spontaneous prophage induction, have been described (337). The cyclic AMP-requiring mutants of *S. marcescens* are particularly interesting because, in the absence of cyclic AMP, the cells are not only deficient in lipase secretion but they are also nonmotile and morphologically deranged so that they frequently form filaments (336). Similar pleiotropic mutations are common in *B. subtilis* and simultaneously result in a lack of flagella, altered morphology, and abnormal exoenzyme synthesis (8, 170, 288). It is possible that an undefined small molecule has a regulatory role in bacilli similar to that of cyclic AMP in *S. marcescens*.

CONCLUDING REMARKS

Our understanding of the process of exoenzyme synthesis in procaryotes, and in particular in the bacilli, has undoubtedly increased in the 13 years since the last review of this kind (247). However, detailed knowledge of the mechanisms governing the expression of exoenzyme genes is still lacking, and many fundamental questions are still unanswered. The nature of exoenzyme induction and repression and the repression of exoenzyme synthesis during exponential growth are often observed, but the mechanisms are unknown. Direct evidence for a membrane location for exoenzyme-synthesizing ribosomes is still lacking, and, although the vectorial discharge of the nascent polypeptide chain from the 50S ribosomal subunit through the membrane forms an attractive hypothesis, it is largely unsubstantiated. Finally, we do not understand how the enzyme traverses the cell wall to become completely extracellular.

There are many difficulties that complicate investigations into the control of extracellular enzyme synthesis and are largely responsible for our ignorance. Experiments that would be relatively simple when using *E. coli* are virtually impossible in *B. subtilis*. Merodiploids of *B. subtilis* are difficult to construct and, in the absence of indigenous episomes and high-frequency transducing phages, the isolation and purification of specific pieces of the genome in high quantity is technically difficult. Thus, genetic complementation and the in vitro transcription systems that have proved to be invaluable in the elucidation of many aspects of gene expression in *E. coli* are presently not available for the study of exoenzyme synthesis in bacilli. It is hoped that as the variety of techniques increases and interest in the bacilli from both academic and industrial standpoints develops many of the outstanding questions will be answered in the near future.

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